Universidade de Lisboa

Faculdade de Medicina de Lisboa

HIV Infection

and

Gut-Associated Lymphoid Tissue

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Doutoramento em Medicina

Imunologia Clínica

A impressão desta dissertação foi aprovada em Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 27 de Janeiro de 2015.

Dissertação apresentada à Faculdade de Medicina de Lisboa

para obtenção do grau de Doutor em Medicina

A presente dissertação foi realizada na Unidade de Imunodeficiência Humana e Reconstituição Imune do Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa

Bolsa para programa de Internato/Doutoramento da Fundação para a Ciência e Tecnologia (SFRH/SINTD/60108/2009)

To Francisco and Sérgio

they were always here

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ACKNOWLEDGMENTS

First, I would like to thank my mentor Professora Doutora Ana E. Sousa for the support and incentive through the last four years.

I would like to thank everyone in the Lab where this work was done, but especially Russell Foxall with whom I did this work and that guided me through the way, Rita Tendeiro and Adriana Albuquerque that first helped when I started at the lab, Ana Rita Pires for all the time dedicated to histology, Paula Matoso for the shared experience on gene expression and finally to Iris Caramalho and Vânia Silva for all the support, enthusiasm and help in the resolution of problems.

I also would like to thank to Catarina Mota and Susana Lopes da Silva that took this walk with me. Their mutual support kept me going.

Then, I have to thank to all the clinical departments involved in this work, including their directors, clinicians and patients, namely Infectious Diseases, Gastroenterology and Pathology departments of Centro Hospitalar Lisboa Norte, Hospital de São Bernardo and to the Infectious Diseases Department of Hospital Professor Doutor Fernando Fonseca. Without them this work would not have been possible. I would like to give a particular thanks to all the clinicians that directly participated in this work, particularly to Dr. Luís Correia that collected most of the gastrointestinal biopsies, Dra. Cristina Ferreira that evaluated all the biopsies, Dr. José Poças for his efficiency and persistence in recruiting patients for this study, Professora Emília Valadas, Dra. Carla Santos, Dr. Robert Madura, Dra. Joana Fernandes, Dr. Nuno Janeiro, Dr. Sérgio Paulo and Dra. Patrícia Pacheco that included their patients in this work.

I also have to give a special thanks to Dra. Eduarda Lourenço, my clinical mentor, that always pushed me to new journeys and to Professor Rui Victorino that encouraged me to pursue this work.

Because a PhD is a hard, long journey I have to thank to all my friends, but especially Laura, as well as to Vasco and Mara, after 10 years of absence they were still there, and to the new friends that appeared along the way and are around in spite of my craziness.

At the end, and these words are not enough, all my thanks to my family that waited for me, Lia Ledo that kept my feet on the ground and to Sérgio and Francisco that lighten my days.

ABBREVIATIONS

Ag - Antigens

AGM – African green monkeys

AhR – Aryl hydrocarbon receptor

AIDS – Acquired Immunodeficiency Syndrome

ART – Antiretroviral treatment

CTL – Cytotoxic CD8 T-cell

DCs – Dendritic cells

EndoCAb – Endogenous endotoxin-core antibody

FAE – Follicle associated epithelium

FICZ - 6-formylindolo[3,2-b]carbazole

FOXP3 - Forkhead box P3

GALT – Gut Associated Lymphoid Tissue

HIV – Human Immunodeficiency Virus

IBD – Inflammatory bowel disease

ICAM – Intercellular adhesion molecule

IEL – Intra-epithelial lymphocytes

I-FABP – Intestinal Fatty Acid Binding Protein

IL – Interleukin

ILF – Isolated lymphoid follicle

ILC – Innate lymphoid cells

ILS – Isolated lymphoid follicles

LP – *Lamina propria*

LPS – Lipopolysaccharide

LT – Lymphotoxin

LTi – Lymphoid tissue inducers

LTNP – Long-term non-progressors

LTo – Lymphoid tissue organizers

MadCAM - Molecular vascular addressin cell adhesion molecule

NK – Natural Killer

pDC – Plasmacytoid dendritic cells

PP – Peyer's patch

RA – Retinoic acid

RMs – *Rhesus Macaques*

ROR - Ror-orphan receptor

sCD14 – Soluble CD14

SM – Sooty mangabeys

TLR - Toll-like receptors

Treg – Regulatory T-cells

Th17 – IL-17 producing CD4+ T-cells

Th22 – IL-22 producing CD4+ T-cells

VCAM – Vascular adhesion molecule

ZO-1 - *Zonula Occludens*

SUMMARY

HIV-1 disease progression is driven by chronic hyper-immune activation, a process intimately linked with severe mucosal CD4 T-cell depletion throughout the infection's course. The failure to fully re-establish mucosal homeostasis upon effective antiretroviral treatment (ART), and the consequent chronic inflammation, is also linked to morbidity despite viral control.

This work aimed to investigate mechanisms underlying recovery of gut homeostasis in HIV-1 treated infection, and to study, for the first time, the mucosal impact of HIV-2 infection, an attenuated form of HIV/AIDS.

We focused the study of long-term treated HIV-1 infected individuals on IL-22, a cytokine essential for intestinal epithelial integrity, and found significant depletion of IL-22-producing CD4 T-cells (Th22) in the sigmoid. However, mucosa was not disrupted, and there was a preserved expression of IL-22-dependent epithelial genes, as well as of innate lymphoid cells able to produce IL-22 (ILC3), thereby implicating this subset in gut homeostasis in HIV-1 infection.

Conversely, long-term HIV-2-infected adults maintained mucosal CD4 T-cells irrespectively of ART, and featured no imbalances of CD4 T-cell subsets. The ILC3 compartment was also preserved, as well as the mucosal structure, despite evidence of local viral replication. Importantly, there was increased local production of chemokines, which supports a balanced CD4 T-cell recruitment to counteract cell loss. Of note, we found mucosal CD4 T-cell depletion with gut disruption in a young adult with HIV-2 infection acquired through vertical transmission, suggesting that HIV-2 infection early in infancy may be associated with loss of gut integrity. Nevertheless, there was a full recovery upon 5 years of ART.

In conclusion, these studies indicate the importance of targeting gut homeostasis to improve HIV/AIDS outcome, highlighting the contribution of ILC3 and IL-22, and demonstrating for the first time that the protracted course of HIV-2 infection is associated with lack of gut disruption.

Key words: HIV/AIDS; HIV-2; Gut associated lymphoid tissue; mucosal homeostasis; IL-22.

SUMÁRIO

O curso clínico da infecção VIH-1/SIDA é determinado pela hiperactivação progressiva do sistema imune devida em parte à depleção de linfócitos T CD4 da mucosa intestinal. Esta inicia-se na fase aguda, e só recupera parcialmente após terapêutica antiretroviral (TAR), contribuindo assim também para a morbilidade em indivíduos tratados.

O objectivo deste trabalho foi investigar os mecanismos que favorecem a homeostasia intestinal na infecção VIH-1 após TAR e, na infecção VIH-2, um modelo natural de doença VIH/SIDA cujo impacto intestinal nunca tinha sido investigado.

O estudo de indivíduos VIH-1 tratados centrou-se na IL-22, citoquina fundamental para a manutenção da integridade epitelial, e demonstrou perda significativa de células T CD4 produtoras de IL-22 (Th22). No entanto, a integridade epitelial e a expressão de genes epiteliais dependentes de IL-22 estava mantida, assim como a frequência de células linfóides inatas produtoras de IL-22 (ILC3), suportando um papel central das ILC3 na manutenção da homeostasia intestinal na infecção VIH-1.

Em contraste, demonstrámos que a infecção VIH-2 prolongada se associava à preservação de linfócitos T CD4 na mucosa, incluindo Th22, independentemente da TAR, e que esta se acompanhava de aumento da produção local de quimocinas envolvidas na migração intestinal destas células, sugerindo um recrutamento aumentado. Documentámos também manutenção de ILC3 e da integridade intestinal apesar de replicação viral local. Contudo, a aquisição de infecção VIH-2 no início da vida num contexto de imaturidade imunológica pode associar-se a perda de integridade intestinal e de linfócitos T CD4, embora recuperáveis após TAR, tal como ilustrámos no estudo longitudinal de um jovem adulto.

Em conclusão, a homeostasia intestinal constitui um alvo terapêutico importante na infecção VIH/SIDA, tendo os nossos estudos salientado o contributo das ILC3 e da IL-22, e demonstrado pela primeira vez que, o curso menos agressivo da infecção VIH-2 se associa a uma preservação da mucosa intestinal.

Palavras-chave: VIH/SIDA; VIH-2; Tecido linfoide associado à mucosa intestinal; homeostasia da mucosa; IL-22.

SUMÁRIO EXTENSO

A infecção pelo Vírus de Imunodeficiência Humana (VIH) tipo 1 constitui um dos mais graves problemas de saúde mundial, tendo sido responsável, desde a descrição dos primeiros casos em 1981, pela morte de mais de 36 milhões de pessoas. O curso clínico desta infecção associa-se a uma perda progressiva de linfócitos T CD4 circulantes, levando nas fases avançadas à síndrome de imunodeficiência humana adquirida ou SIDA, caracterizado pelo aparecimento de infecções oportunistas responsáveis pela morte dos indivíduos infectados na ausência de tratamento. Apesar da introdução de terapêutica antirretroviral (TAR) eficaz em 1996, os doentes infectados pelo VIH-1 têm ainda uma maior morbilidade quando comparados com a população em geral.

A diminuição progressiva do número de linfócitos T CD4 circulantes está relacionada não só com efeitos citopáticos directos ou indirectos do vírus, mas também com um estado de hiper-activação crónica do sistema imune que está subjacente à patogénese desta infecção. São vários os factores contributivos para este estado inflamatório crónico, tendo nos últimos anos sido dado uma particular relevância à perda de homeostasia da mucosa intestinal.

A disrupção da mucosa intestinal ocorre precocemente durante a infecção pelo VIH-1, contribuindo para este estado inflamatório crónico através da perda da integridade epitelial intestinal e do consequente aumento de translocação de produtos microbianos.

O sistema imunitário associado ao intestino é constituído por várias estruturas linfóides organizadas, tais como as placas de Peyer ou os folículos linfóides, e por células isoladas que patrulham a lâmina própria da mucosa intestinal, nomeadamente linfócitos T CD4. Na realidade, a mucosa intestinal é o local do organismo onde se encontra o maior número de linfócitos T CD4. As células T CD4 capazes de produzirem interleucina (IL)-17, células Th17, e IL-22, células Th22, são particularmente relevantes dado o papel destas duas citocinas no controlo da flora

microbiana. A IL-22 tem ainda um papel não-redundante na manutenção da integridade da mucosa, através da estimulação da proliferação epitelial e produção de péptidos antimicrobianos. Por outro lado, dada a necessidade de manter um ambiente de tolerância para permitir uma eficaz absorção de nutrientes, uma elevada percentagem de células T CD4 têm função reguladora (Treg), sendo identificadas pela expressão do factor de transcrição FOXP3. A regulação da inflamação e homeostasia na mucosa intestinal é assegurada por vários outros tipos de células, tendo sido reconhecido nos últimos anos o papel fulcral das células linfóides inatas (ILC). Estas últimas são capazes de se diferenciar em vários subgrupos, sendo as que produzem IL-22 pertencentes às ILC3.

A perda de homeostasia da mucosa intestinal na infecção pelo VIH-1 está associada à marcada depleção dos linfócitos T CD4 na lâmina própria, predominantemente das células Th17, que ocorre desde a infecção aguda e se mantém durante toda a história natural da infecção. A TAR embora muito eficaz no controlo da replicação viral, não restabelece na totalidade a população de linfócitos T CD4 da mucosa intestinal, mesmo após vários anos de virémia indetectável.

Neste trabalho colocámos a hipótese de que esta disrupção da mucosa intestinal na infecção pelo VIH-1, mesmo em doentes tratados, pudesse estar relacionada com a diminuição da produção de IL-22. Para avaliar a produção na mucosa intestinal de IL-22 e o seu contributo para a integridade desta, foram estudadas xviiopsias intestinais de 17 doentes infectados pelo VIH-1 sob TAR e comparadas com as de 20 indivíduos saudáveis com idade semelhante, através da colheita de biópsias da sigmóide durante colonoscopias realizadas no âmbito de rastreio de cancro colo-rectal. Todos os doentes VIH-1 estudados estavam avirémicos, e tinham uma mediana de tempo sob TAR de 8 anos e recuperação completa do número de linfócitos T CD4 circulantes. Verificou-se uma diminuição significativa de células Th22, quer no que diz respeito à sua frequência (percentagem do total de células T CD4, avaliado por citometria de fluxo da suspensão celular efectuada a partir de 10 biópsias) quer em relação ao seu número (número de células duplamente positivas para IL-22 e CD3, contadas em biópsias marcadas por imunofluorescência). Embora se verificasse uma redução da produção total de IL-22

na mucosa intestinal, a produção de IL-22 pelas ILC3, definidas pela expressão de cKit e NKp44, estava mantida, assim como a sua frequência. Simultaneamente, não se encontraram alterações no nível de expressão de genes relevantes para a indução de IL-22, nomeadamente IL-23, IL-6, IL-1β ou TGFβ. De facto, apesar da redução significativa das células Th22, a preservação da produção de IL-22 pelas ILC3 associou-se a uma preservação da barreira epitelial avaliada por imunohistoquímica, bem como da expressão de genes dependentes da IL-22 (ex: mucina 1, mucina 13 ou S100A9) que são essenciais para o controlo do microbioma intestinal. Em resumo, este estudo enfatiza o papel das células ILC3 para a manutenção da homeostasia da mucosa intestinal num contexto em que existe diminuição de Th22, podendo no futuro ser alvo de terapêuticas que visem alcançar a integridade da mucosa intestinal de forma a diminuir a inflamação persistente em doentes tratados.

A activação imunitária é também um reconhecido motor da perda de linfócitos T CD4 na infecção pelo VIH-2. Este retrovírus tem uma homologia de cerca de 60% com o VIH-1, tendo o mesmo tipo de alvos celulares e o mesmo espectro clínico. No entanto, a infecção pelo VIH-2 tem um curso clínico significativamente mais indolente, caracterizando-se por uma perda lenta de linfócitos T CD4 e virémia reduzida ou indetectável. Tendo em conta estas características, esta infecção é considerada um modelo humano de doença "atenuada", sendo o estudo dos mecanismos subjacentes à sua mais lenta progressão para SIDA fundamental para a compreensão da patogénese da infecção pelo VIH em geral. Até ao momento não foram realizados estudos para avaliar o impacto da infecção pelo VIH-2 na mucosa intestinal. Dado o curso clínico mais benigno, é plausível colocar a hipótese de a mucosa intestinal nos doentes infectados por VIH-2 estar preservada.

O segundo objectivo deste trabalho foi estudar pela primeira vez o impacto da infecção pelo VIH-2 na homeostasia da mucosa intestinal.

Tivemos oportunidade de efectuar o estudo longitudinal de um doente infectado por VIH-2 no primeiro ano de vida, que apresentava, aos 18 anos, quando foi feito o diagnóstico, critérios de SIDA. A investigação de diarreia crónica e atraso significativo do crescimento revelou a existência de úlceras rectais profundas, em

associação com perda significativa de linfócitos T CD4 na lâmina própria, aumento de células Treg e diminuição de células produtoras de IL-17. Simultaneamente, foi comprovada a existência de replicação do VIH-2 na mucosa intestinal, avaliada pela marcação positiva de células para a proteína viral Gag. A perda de homeostasia da mucosa intestinal associava-se a aumento de produtos microbianos circulantes e inflamação crónica. Foi introduzida TAR, tendo-se observado uma recuperação total da homeostasia da mucosa intestinal ao longo de cinco anos, o que contrasta com a limitada recuperação habitualmente documentada nos indivíduos infectados pelo VIH-1. O estudo longitudinal deste doente com infecção pelo VIH-2, adquirida por transmissão vertical, demonstra a possibilidade de replicação viral na mucosa intestinal, em associação com perda significativa de linfócitos T CD4, independentemente de baixa virémia.

Para a avaliação do impacto da infecção crónica pelo VIH-2 em adultos foi estudado um grupo de 13 doentes, sete dos quais sob terapêutica antiretroviral, com indicação para colonoscopia de rastreio do cancro colo-rectal. Tal como previamente demonstrado verificaram-se no grupo com infecção pelo VIH-2, níveis elevados de activação imunitária, quando comparados os de indivíduos saudáveis e alguns doentes apresentavam redução significativa de linfócitos T CD4 circulantes (entre 250 e 300 células/μL).

A avaliação da estrutura da mucosa intestinal foi efectuada através de análise histológica detalhada e de quantificação da expressão de mucinas e péptidos anti-microbianos, não tendo sido encontrada nenhuma diferença entre os grupos. A permeabilidade da mucosa intestinal depende da integridade das junções celulares. Ao contrário do descrito para a infecção pelo VIH-1 verificámos ausência de alterações nos níveis de expressão de ZO-1, uma das proteínas das junções celulares, nos indivíduos infectados pelo VIH-2. Desta forma, concluímos que a homeostasia da mucosa intestinal estava preservada.

Salienta-se ainda que foi possível demonstrar em 5 dos 13 doentes infectados pelo VIH-2 a existência de replicação viral na mucosa intestinal, apesar das virémias serem indetectáveis. Documentou-se também expansão de linfócitos T CD8

produtores de Interferão-γ (IFNγ) na mucosa, de acordo com algum grau de replicação viral local. No entanto, a avaliação de linfócitos T CD4 na mucosa intestinal não mostrou alterações comparativamente com 20 indivíduos saudáveis, não existindo diferenças quer no seu número ou frequência, quer no que diz respeito à produção de IL-22, IL-17 ou IFNγ. Salienta-se ainda que o número e frequência de Treg era normal. Estes achados contrastam com os dados obtidos no estudo longitudinal do doente com infecção pelo VIH-2 adquirida no primeiro ano de vida, sugerindo que na idade adulta o impacto do VIH-2 na mucosa intestinal é reduzido.

Com o objectivo de encontrar possíveis mecanismos que explicassem a manutenção do número de linfócitos T CD4, particularmente Th17 e Th22, na mucosa intestinal, apesar de infecção viral persistente, foi demonstrado que a expressão local de vários genes associados à homeostasia destas populações celulares, nomeadamente IL-23, IL-6, IL-1β ou TGFβ, tinha níveis semelhantes aos dos controlos saudáveis. Salienta-se porém o aumento significativo da expressão de CCL20 e CCL28, quimoquinas relevantes para a migração de células T CD4 para a mucosa, o que sugere que existe um processo activo de recrutamento linfocitário para assegurar a manutenção do número destas células

Em resumo, os resultados obtidos em adultos infectados pelo VIH-2 demonstram um impacto diferencial deste vírus na mucosa intestinal comparativamente com o VIH-1. É plausível que a manutenção da integridade da mucosa intestinal na infecção pelo VIH-2 explique o ritmo lento de progressão da activação imunitária e consequentemente de progressão para SIDA.

Os nossos resultados reforçam a importância do estudo da infecção VIH-2 como modelo de manutenção de homeostasia da mucosa intestinal na presença de uma infecção viral persistente, e o seu potencial para identificar novos alvos terapêuticos com implicações para a infecção VIH-1/SIDA e para outras situações clínicas associadas a inflamação intestinal crónica.

CHAPTER 1. INTRODUCTION

1.1. The gut: the largest human barrier

The gut mucosa is the largest body surface contacting the outside environment, functioning as a selective gate where nutrients are allowed to pass and foreign organisms are kept at bay. Far from being a simple physical barrier, there is an active interplay between the foreign elements: microbiota, and human cells that compose the gut (epithelial, stromal and immune cells). This interaction shapes both ecosystems in a continuous process starting after birth, which determines the overall health of humans[1, 2].

Given its relevance in many clinical contexts, the investigation of gut homeostasis and possible therapeutic targets centered within this organ currently represents a cutting edge field of research. Major advances have occurred in recent years, showing that many physiological processes depend on the crosstalk between gut commensal flora and the gut-associated lymphoid tissue (GALT). This is true not only for disease processes limited to the gastrointestinal tract, like inflammatory bowel disease[3], but also for systemic diseases like multiple sclerosis[4, 5] or rheumatoid arthritis[6], as well as for HIV infection, the focus of this thesis.

1.1.1. The gate: a specialized epithelial layer

The gastrointestinal tract is a very large structure with several compartments, each with different functions (digestion, absorption of nutrients and water). The lining of the gut tube derives from the endoderm, and during embryonic development it expands several times the length of the body in order to ensure an adequate absorption of nutrients and water in adult life, as reviewed by Noah et al[7]. The wall of this long specialized structure is composed of the mucosa that contains the epithelial layer and lamina propria, below which lies the sub mucosa, then the muscular layer and finally the serosa[8]. Additionally, in order to prepare for bacteria colonization after birth, lymphoid structures develop along the intestinal wall[9].

As well as its role in digestion and absorption of nutrients, the epithelium, provides, in conjunction with GALT, an important line of defense that controls the

commensal flora and limits pathogenic invasion, as recently reviewed by Peterson et al[10]. This single layer of cuboidal epithelial cells is organized in a tridimensional structure of villi and crypts in the small bowel and crypts in the large bowel[11],which both maximizes the available area for absorption and contact with the gut lumen environment (Figure 1). The most abundant cells, in both small and large bowel are absorptive enterocytes and mucus-secreting goblet cells. These are continuously being renewed at the tip of the villous (small intestine) or crypt (colon) by enterocytes derived from epithelial stem-like cells localized at the base of the crypts[12]. In the small bowel, the basal crypt area contains antimicrobial peptide-secreting Paneth cells[13] that facilitate the survival of epithelial stem cells.

The relative impermeability of the epithelium is maintained by tight junctions, formed by proteins of the claudin and *zon occludens* families, located at the latero-apical face of the membrane. These structures are fundamental for maintaining the tight adhesion of epithelial cells, and their disruption is one of the mechanisms used by several pathogenic agents to invade gastrointestinal mucosa[14].

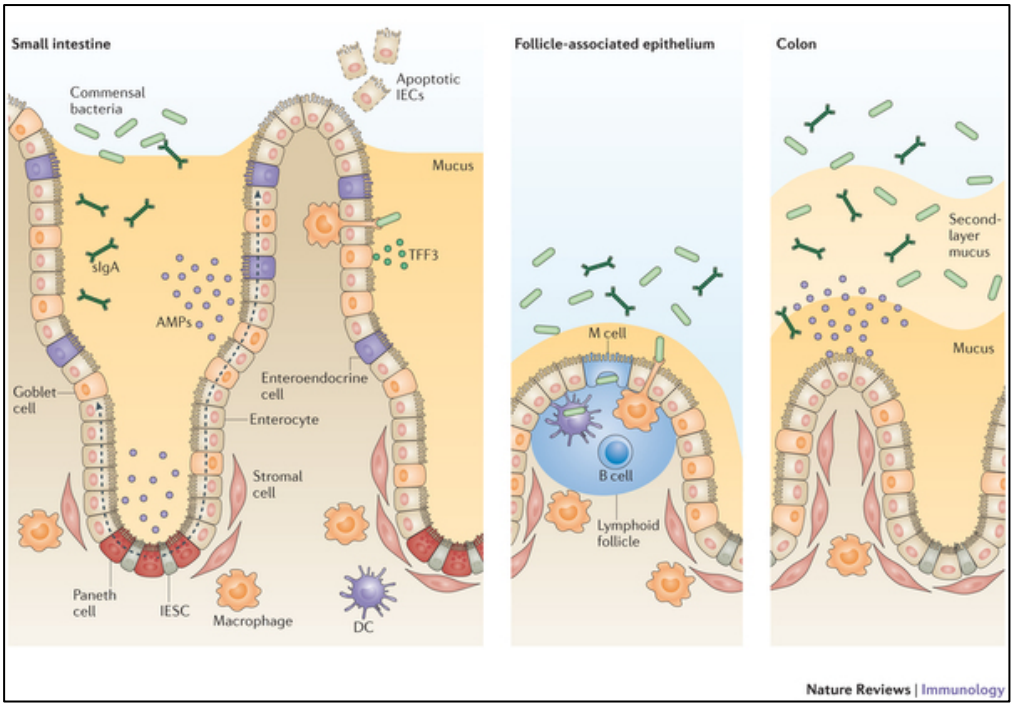

Figure 1. Intestinal epithelial structure in small intestine and colon. Schematic representation of small intestine and colon. *Adapted from Peterson et al. Nature Rev. 2014[10].*

As previously mentioned, and, though once considered simply as a physical barrier with selective absorptive capacities, epithelium is now understood to form part of the communication network between the environment and the immune system, shaping immune cell responses. Epithelial cells sense microbial constituents through pattern recognition receptors, like Toll-like receptors (TLR) or NOD like receptors[15] and G-protein coupled receptors. Recognition of bacterial signals through TLR is essential for the development of mucosal immune responses and maintenance of normal microbial commensal communities[16].

Nevertheless, to avoid over stimulation by bacteria in the gut lumen, epithelial cell expression of these molecules is physically restricted to the cytoplasm and/or the basolateral portions of the membrane. For instance TLR5, the receptor for bacterial flagellin, is expressed only on the basolateral membrane. Thus the presence of flagellated bacteria beneath the epithelial surface is required to trigger this receptor, a process that induces the expression of interleukin (IL)-8 and MIP3α[17], and CCL20[18] which subsequently recruit immature dendritic cells[19] and T-cells to the mucosa, initiating an immune response. Furthermore, TLR2 and TLR4 are expressed in reduced amounts in steady state conditions, and are mainly restricted to the crypts[20, 21], aiding epithelial hypo-responsive to gram-positive bacteria[22] and the gram-negative product lipopolysaccharide (LPS) which is highly abundant in the gut lumen. Epithelial cells also express TLR9 that recognizes specific sequences of bacterial DNA. However, *in vitro* ligation with its ligand does not induce an inflammatory response[23] if engaged on the apical surface of epithelial cells[24], another mechanism by which the epithelium protects itself from over stimulation. The distinction between pathogenic and non-pathogenic bacteria does not only depend on where the microbe is sensed, as discussed above, but also on the levels ATP produced by luminal bacteria, a process that modulates epithelial cells responsiveness to TLR ligands[25].

On responding to luminal bacteria, epithelial cells can induce either tolerogenic or inflammatory responses. They express MHC-class II molecules, and can present antigens to T-cells, although this process usually results in T-cell anergy[26], thereby contributing to mucosal tolerance to gut microbes. Moreover, epithelial cells

secrete TGFβ and retinoic acid[27, 28], inducing, via dendritic cells, the maturation of regulatory T-cells, a subset highly abundant in the mucosa, as explained later.

Above the epithelial layer there is a stratified mucus layer composed of mucins, antimicrobial peptides and antimicrobial immunoglobulins, which is at its thickest in the stomach and colon and thinner in the small bowel, to facilitate this region's absorptive function (reviewed by McGuckin et al[29]). The inner surface of the mucosal layer is dense, and devoid of commensal bacteria, whereas the outer layer is loose and supports the attachment of commensal flora that feed on the sugars composing this layer[30]. The metabolism of these sugars produces butyrate, which feeds back to epithelial cells and increases their production of mucins[31]. The mucin and antimicrobial peptide composition of this layer differs along the gastrointestinal tract[29], and the combination of several of these proteins helps increase the viscosity of this barrier. In addition, these molecules, in conjunction with trefoil factors, facilitate repair of intestinal breakage resulting from injury, through a mechanism involving epithelial cell migration[32].

Overall, the epithelium, continually renewed, contributes to a controlled communication between the environment and the immune system, in addition to its non-redundant absorptive and digestive functions.

1.1.2. GALT: the immune cells that defend the gate

Underneath the epithelium lie different well-defined lymphoid structures: the tonsils, in the mouth; and the Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and cryptopaches in the intestine. Apart from these well-organized structures, where the priming of immune cells occurs, GALT is also composed of lymphoid cells scattered along the epithelial layer (intra-epithelial lymphocytes – IEL) and the lamina propria (LP). At the crossroads with the systemic immune system, lie the specialized lymphoid structures where lymphocytes mature: the mesenteric lymph nodes[8].

At birth, and before gut colonization, PPs can already be found lining the small intestine wall. These are macroscopic lymphoid structures found in the small intestine, particularly ileum[33]. They are located in the lamina propria and extend into the submucosa, and are composed of a specialized epithelial layer, called follicle-associated epithelium (FAE), that contains enterocytes, microfold cells (M cells) and is devoid of brush border and mucus layer, allowing antigens (Ag) to pass to the sub-epithelial dome where dendritic cells take them up. Follicle-like regions are located below this structure, containing T and B cell areas similar to those found in lymph nodes[34].

The development of these structures parallels the development of lymph nodes, relying on interactions between hematopoietic-derived and mesenchymal cells. Most of the detailed knowledge about mammalian embryonic development of PPs derives from murine studies[35]. At week 12 of mouse embryonic life, hematopoietic-derived cells, called lymphoid tissue inducers (LTi cells, $CD3^{-}CD4^{+}cKIT^{+}IL\text{-}7R\alpha^{+}\alpha4\beta7^{+}$) populate the gut mucosa. The development of these cells depends on, amongst other factors, the transcription factors Ror-orphan receptor(ROR)γt and Id2. LTi are attracted to the mucosa by CXCL13 and produce lymphotoxin (LT) α1β2 in response to IL-7 stimulus. Next, LT induces lymphoid tissue organizer (LTo) cells, mucosa mesenchymal cells, to express vascular adhesion molecule (VCAM) 1, intercellular adhesion molecule (ICAM)-1, IL-7, CCL21, CCL19 and CXCL13, that attracts more LTi and contributes to their local survival, creating a positive feed-back loop[36, 37]. In addition, animal models have also demonstrated the requirement for a third cell-type in Peyer's patch development: a hematopoietic-derived cells called lymphoid tissue initiator (LTin cells)[38, 39]. The interplay between these three cell types, results in the formation of the PP primordia along the mouse gut mucosa in a stochastic manner.

PP colonization by mature B and T-cells occurs via the high endothelial venules in a molecular vascular addressin cell adhesion molecule (MadCAM)1 dependent fashion. The subsequent formation of lymphoid follicles occurs in mice at the time of birth and is independent of the presence of microbes.

After gut colonization by commensal flora, the GALT matures, and other lymphoid structures, namely isolated lymphoid follicles (ILFs) are formed[40, 41]. ILFs represent mature forms of cryptopatches, an embryonic structure formed by mesenchymal and LTi cells. Their number and localization is highly variable from species to species and is dependent on gut colonization by microbes, as germ-free mice have a reduced number of ILF[42]. These structures complement the role of PPs by promoting IgA class switching in B cells, attracted to ILFs by dendritic cell-secreted CXCL13[43]. In a process driven by RANKL, the epithelium above ILFs also changes, and acquires some similarities to that found in PPs, including the presence of M cells and decreased numbers of goblet cells[44]. As previously mentioned IgA is secreted into the gut lumen and covers almost the entire epithelial surface, contributing to its barrier function. B cell class switching depends upon Ag presentation by dendritic cells, together with the help of CD4 T-cells in the ILF[45].

Apart from their role in the function of the specialized lymphoid structures found in the gut mucosa, T-cells, previously primed in mesenteric lymph nodes, patrol the intestinal lamina propria. Their ingress into the lamina propria is regulated by selective expression of cellular adhesion molecules on T-cells and the vascular endothelium of the small and large intestine[46], resulting in the immune compartmentalization of these sites[47] . After priming in the small intestinal draining lymph nodes, T-cells acquire the expression of α4β7 that recognizes MadCAM-1 expressed on the lamina propria endothelial vasculature[48]. CCR9 expression is also a fundamental requirement for homing to the proximal intestinal tract (duodenum and jejunum) where high levels of CCL25 are expressed[49, 50]. CCL25 levels are much lower in the colon, where T-cell trafficking seems to rely mainly on α4β7 expression and the CXCR4-CXCL12 axis[46], although the role of this chemokine receptor/ligand pair is yet to be proven. Migration to the gut mucosa is also dictated by the expression of CCR6 on several CD4 subsets[51]. This receptor recognizes the chemokine CCL20, which is highly abundant in the gut mucosa. The relative importance of each of these pathways is hard to ascertain, and the overall migration to the mucosa is probably the result of the combination of these signals.

CD4 T-cells are one of the most abundant cell types in the gut mucosa, and importantly it is in the gut mucosa that lies the largest pool of these cells.

Upon priming in the lymph nodes, CD4 T-cells acquire a memory and activated phenotype, expressing high levels of CCR5 and CXCR4[52], and acquire the transcriptional program that dictates their function in the gut mucosa. The mechanisms that sustain their survival in the gut mucosa are not completely understood, but seem to depend upon OX40 and CD30 signals, as mice T-cells deficient in these molecules are able to migrate to the mucosa but do not persist there[53]. Although less frequent than CD4 T-cell in the lamina propria, CD8 T-cells are the most common T-cell in the epithelial layer. Finally, γδ T-cells can also be found in the gut lamina propria, and are involved in maintaining gut homeostasis and also help provide protection against several pathogens[54].

1.1.2.1. Th17 and Th22 patrol lamina propria and guarantee epithelial homeostasis

Naïve CD4 T-cells acquire different specializations upon priming by dendritic cells in the lymph nodes and the integration of different environmental signals. One of the most relevant CD4 T-cell subset in the lamina propria is the one that produces IL-17, in both healthy and disease states. This Th17 subset is critical to the defense against several pathogens, through the recruitment of neutrophils[55] and induction of antimicrobial peptides[56]. IL-17A is a pro-inflammatory cytokine, inducing neutrophil recruitment[55], epithelial proliferation and chemokine production (e.g. CXCL2, CXCL5, CXCL8, GM-CSF)[57]. Its presence is critical for defense against pathogens, namely *Candida albicans*[58, 59], *Bacteroides fragilis*[60], and *Klebsiella pneumoniae* in the lung mucosa[61, 62]. Th17 cells have also been shown to produce together with IL-17A, IL-22 and IL17F, and to express CCR6, the receptor for IL-23, as well as CD161[63]. The induction of Th17 cells depends of RORC expression and the presence of several cytokines, in particular IL-1β, IL-6, IL-23 and small amounts of TGFβ and IL-2[64]. All of these factors are abundant in the gut mucosa, being produced by dendritic and epithelial cells, as well as neutrophils[55] in response to commensal flora, creating an environment favourable to Th17 induction.

Although IL-22 and IL-17 production were originally considered to be produced in unison in the majority of mucosal responses, the former can occur independently of IL-17, and recently a different CD4 T-cell subset, able to uniquely produce this cytokine (Th22), was identified[65].

In common with IL-17, IL-22 production depends upon the expression of RORC, although, in human cells, this is not sufficient for its induction. In fact, human naïve CD4 T-cells transduced with RORC, unlike their murine counterparts, do not express IL-22[66]. Its production is also triggered by IL-23, secreted by dendritic cells and macrophages, in response to epithelial danger signals, such as IL-1 and IL-6[67]. In contrast to IL-17, the expression of the aryl hydrocarbon receptor (Ahr) is fundamental to IL-22 induction (Figure 2)[68]. This molecule was initially identified as a mediator in the toxicity of xenobiotics such as 2,3,7,8-tetrachlorobenzo-p-dioxin (TCDD). Subsequently, endogenous ligands of Ahr were identified, the most important of which are metabolites of tryptophan metabolism such as 6-formylindolo[3,2-b]carbazole (FICZ). In addition, IL-22 provides a fundamental link in the communication between immune cells and the epithelium. It has been implicated in mucosal tissue homeostasis, local inflammatory processes and defense against pathogens, as illustrated by its non-redundant protective role against *Citrobacter rodentium* gut infection in murine models[69, 70].

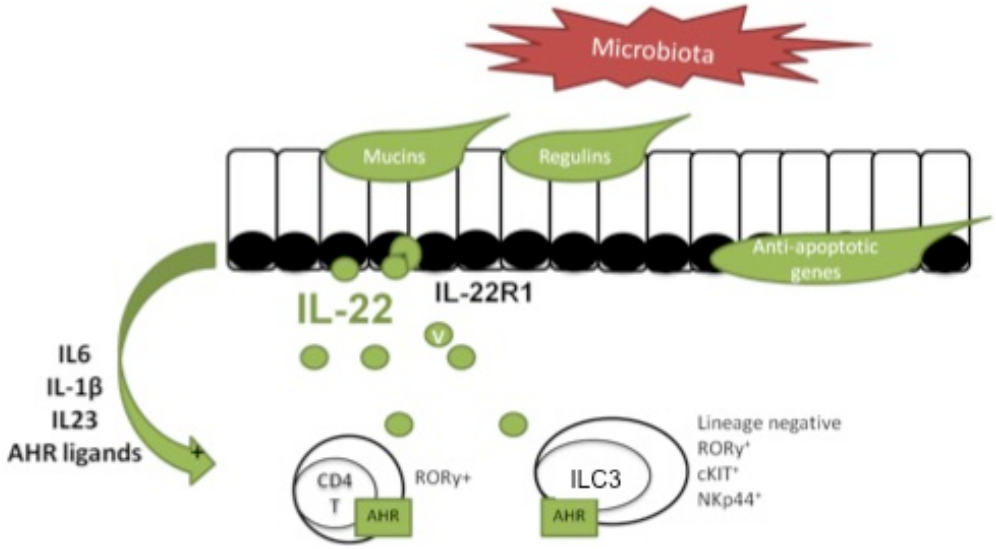

Figure 2. Mucosal IL-22 production. IL-22 produced by CD4 T-cells and type 3 innate lymphoid cells (ILC3) signals in the epithelium leading to the production of mucins and antimicrobial peptides. Its production is induced mainly by IL-6, IL-23 and IL-1β, as well as several AhR ligands.

IL-22 targets epithelial cells, which express high levels of its receptor (IL-22R), a heterodimer consisting of the IL10R2 and IL22R1chains[71]. As it is only expressed on non-hematopoietic cells, it provides one-way communication between immune cells and epithelia. Downstream of its receptor, IL-22 signals via STAT3 and induces the production of protective molecules (e.g. defensins), as well as chemokines that promote T-cell homing[72]. Its role in regulating inflammation has not been completely defined as evidence exists that, depending on the circumstances, it might have a pro[73] or anti-inflammatory role[74]. It also triggers epithelial proliferation, and this, although very important for epithelial repair, has been associated with development of colon cancer[75].

IL-22 is also produced by type 3 innate lymphoid cells (ILC3)[76], which represent the other major source of mucosal IL-22, as discussed below.

Furthermore, several other cell types were shown to be able to produce this cytokine in reduced amounts, namely dendritic cells, NK cells and CD8 T-cells, although their relative contribution to overall mucosa IL-22 availability is still unknown.

Data from murine studies suggest that the source of IL-22 might have implications on its functional impact on the epithelia, and one might speculate that this is related to the specific localization of different cell types.

Given its pro-inflammatory and epithelial proliferation promoting capacity, IL-22 production in the gut mucosa is kept under tight control by several mechanisms, including epithelial cell production of IL-25, a cytokine that forms part of a negative feedback loop controlling IL-17[77] and IL-22 secreting cells, and dendritic cell production of soluble IL-22 receptor (IL-22 binding protein - IL-22BP)[78].

In summary, IL-22 is a fundamental cytokine in the crosstalk between the epithelia and the immune system, thus offering a therapeutic target for the future, with the design of drugs, able to modulate IL-22 secretion, in order to limit intestinal inflammation, as reviewed by Mizoguchi, A et al[79].

1.1.2.2. Inflammation control by Tregs

The gut mucosa must peacefully coexist with an abundant commensal bacterial flora and foreign elements that are generated by feeding. To guarantee this the gut mucosa contains a large pool of regulatory T-cells (Tregs)[80]. These cells, characterized by their expression of the transcription factor FOXP3 (essential for their function and stability), can originate in the thymus or be induced in the periphery from both naïve and memory conventional T-cells[81]. Peripheral induction of Treg and Th17 is apparently linked due to a mutual requirement for TGFβ, which have implications for cell plasticity[82]. In fact, though the presence of low concentrations of TGFβ, together with IL-6 and IL-21, favours Th17 development, a high level of this cytokine induces Treg differentiation[83]. Additionally, low environmental levels of tryptophan in the gut favours Treg over Th17 development. This occurs in the mucosa due to the high levels of expression of indoleamin 2,3-dioxygenase (IDO) by dendritic cells and macrophages. This enzyme deprives neighbouring cells of tryptophan by metabolizing it to kyneurenins. Besides its role in promoting Treg induction, IDO also reduces the proliferative capacity of T and B cells, contributing to the tolerance to gut commensal flora. Moreover, microbe-derived butyrate is an additional inducer of Treg[84].

Thus, the gut mucosa is particularly enriched in induced Tregs, although the relative contribution of induced and thymically-derived Tregs to the maintenance of mucosal tolerance is not completely understood.

1.1.2.3. Spying on the environment: the role of mucosa dendritic cells and macrophages

The effector cells of the adaptative immune system interact with different Ag presenting cells that are continuously sampling gut lumen[19]. Dendritic cells (DCs) are one of the most important types of Ag presenting cells, and they play a fundamental role in both maintaining tolerance and inducing inflammatory responses to pathogenic bacteria, as reviewed by Coombes et al.[85]. These apparently contradictory functions rely on different DCssubsets, with different strategic localizations in the gut mucosa and exposure to a changing environment.

In order to sample antigens from the gut lumen, DCs project dendrites that protrude between epithelial cells. This was demonstrated both *in vitro* and *in vivo* in response to TLR-mediated signaling in epithelial cells[19]. Having collected luminal antigens DCs migrate to mesenteric lymph nodes where they present them to T-cells. T-cells interacting with intestinal DCs in mesenteric lymph nodes are imprinted with the ability to migrate to the gut mucosa. So to maintain a tolerogenic environment, DCs preferentially induce Tregs[85]. This is particularly true for murine CD103+ DCs that were shown to be prone to induce naïve T-cells to become Treg, in a TGFβ and retinoic acid (RA) dependent manner[86]. RA is a by-product of vitamin A metabolism and is mainly produced by DCs. It is not only crucial for Treg differentiation, but low concentrations of this metabolite are also important for Th17 differentiation, through the regulation of epithelial cell production of mucins and consequent regulation of commensal flora, particularly segmented filamentous bacteria[87].

Additionally, DCs can also facilitate the induction of Th17 cells, particularly $CD103^{-}$DCs cells, via their secretion of IL-23. Although DCs are involved in maintaining a tolerogenic environment in the gut, human $CD1b^{+}$ DCs were shown to have a more activated phenotype (higher levels of expression of CD40, CD83, CD86, HLADR) and to produce IL-23 in response to TLR4 and TLR7/8 ligands, as compared to their peripheral blood counterparts[88]. $CD103^{+}CD11b^{+}$lamina propria DCs were also shown to be able to secrete IL-23 in response to TLR5 recognition of bacterial flagellin, promoting the induction of IL-17 and IL-22 production[89]. This more inflammatory phenotype is particularly relevant in guaranteeing the containment of commensal flora, and invasion of the mucosa by pathogenic bacteria and virus.

Although the mechanisms that control the switch between tolerogenic and inflammatory DC are not clearly understood, it is apparent that these cells play a critical role in maintaining mucosal homeostasis, as towel as initiating the adaptive response to pathogenic agents.

In parallel, the mucosal lamina propria is also populated by macrophages that lack CD14 and CD89 expression, and that are also tolerogenic[90-92]. The relative

importance of macrophage vs. dendritic cell antigen presentation and its role in guaranteeing adequate mucosal immune responses is also not completely understood.

1.1.2.4. Innate lymphoid cells: the new commanders

A central role in the control of gut inflammation, and orchestration of immune tolerance to commensal flora has been recently attributed to a group of innate cells, the so-called innate lymphoid cells (ILC). These hematopoietic cells, that share phenotypic characteristics with LTi cells, were first described as a particular subset of natural killer (NK) cells, capable of producing IL-22 and lacking cytotoxic activity[76, 93]. They are characterized by absence of lineage markers (CD3, CD19, CD14, CD16) and by their expression of IL-7Rα (CD127). Given the growing knowledge about different cell types within this group, a new nomenclature was proposed, in which NK cells were re-classified as members of group 1 ILC (ILC1); group 2 ILC (ILC2) produce type 2 cytokines and require RORα for their development, and group 3 ILC (ILC3) include LTi and cells capable of producing IL-22 and IL-17 that, like LTi cells, are dependent of the transcription factor RORγt and ID2 for their development[94]. Recently, a subset of human ILC1, distinct from NK cells but also able to produce IFNγ, with an intra-epithelial localization was identified[95].

ILC3 were shown to be important for the defence against several pathogenic bacteria and at the same time to contribute to the tolerogenic environment of the gut[96]. They are the main innate producers of IL-22[97, 98], which signals to epithelial cells inducing their production of antimicrobial peptides like RegIIIγ, and controlling lumen bacteria, as previously mentioned. These innate cells are preprogrammed to change their cytokine profile from homeostatic to pro-inflammatory in response to pathogen exposure. For instance, in response to *Salmonella* infection, they produce increased amounts of IFNγ in a Tbet-gradient dependent manner[99]. In addition, they are non-redundant for the control of *Citrobacter rodentium* infection, a mouse model of human *Escherichia coli* infection[69].

On the other hand, their presence is also fundamental in controlling inflammatory responses in the mucosa. In RORγt deficient mice, which lack both ILC3 and Th17 cells, containment of gut microflora occurs at the expense of increased numbers of tertiary lymphoid follicles, and high levels of neutrophils and B cell recruitment, which by itself results in damage to the gut mucosa[100].

ILC also have been shown to modulate the survival and function of T and B cells, both through the expression of co-stimulatory molecules, such as OX40L, and the production of relevant cytokines, such as BAFF[101]. The role of ILC3 in the maintenance of mucosal tolerance to gut flora has recently been established in murine models. ILC3 respond to IL-1β secreted by macrophages, in response to commensal flora, by secreting GM-CSF that triggers the expression of IL-10 and RA by macrophages and dendritic cells, thereby facilitating Treg induction[102]. Moreover, ILC3 are known to control Th17 responses to microflora, in a process dependent upon aryl hydrocarbon receptor (AhR) signalling[103]. In contrast, these cells have been implicated in the pathogenesis of inflammatory bowel disease, a combination of disease phenotypes associated with increased levels of inflammation in the small and large intestine.

Regardless of their actual role in health and disease, the increased knowledge about these cells renders them attractive targets for therapies aimed at reconstituting mucosal homeostasis in several clinical situations, as discussed below.

1.1.3. Delicate balance between tolerance and defense in health and disease

The gut mucosa can be viewed as the site of constant pro- and anti-inflammatory processes, an environment fundamental for maintaining its overall homeostasis, starting right after birth.

In fact, following birth, the human gut is colonized with more than a 100 trillion organisms that co-exist with the host in a symbiotic manner[9]. The commensal microflora supplies essential nutrients, digests otherwise indigestible molecules,

promotes angiogenesis and enteric nerve function, forms a protective biofilm that helps defend against pathogenic agents, and ultimately shapes the immune system[104]. The composition of gut microflora is established in two important phases of life: at birth and after weaning from maternal feeding. Even at this early time in life shifts in gut microbiome composition due to cesarean delivery, have been associated with an increased probability of developing disease, such as allergies[105, 106].

Microbe compartmentalization in the gut results from the combination of each microorganisms ability to survive in different gut environments and the compartmentalization of gut immune responses. Outside the epithelial barrier, several compartments can be considered: the lumen, the mucus layer and the apical membrane of epithelial cells, all of which are differentially colonized, with the maintenance of each bacteria in its niche being fundamental to a healthy gut[107]. In a seminal study, Ivanov et al found that *Clostridium*-like species, such as segmented filamentous bacteria, that reside in the mucus layer, interact directly with the epithelium, determining the number of Th17 in the small intestine lamina propria through the stimulation of IL-23 production by monocytes[108]. Antibiotic treatment with agents that preferentially inhibit gram-positive bacteria growth had already been shown to decrease LP CD4 T-cell numbers, as well as influencing the Th17/FOXP3+ cell ratio[109].

The gut microbiome influences not only GALT, but also the systemic immune system. This is illustrated by the impact of a bacterial polysaccharide, produced by the commensal microbe *Bacteroides fragilis*, on the induction of systemic Th1 responses and mucosal regulatory T-cells, which in turn helps determine protection from colitis in a mouse model[41]. Moreover, animal models deficient in genes associated with anti-inflammatory mechanisms (IL-10 or TGFβ) are more susceptible to gut inflammation, a process that is ameliorated or even abrogated on antibiotic administration, or if the mice are housed in a germ-free environment.

The impact of the commensal microflora on the formation and maintenance of GALT and the associated mesenteric lymph nodes has been extensively studied using murine models kept in germ-free conditions.

Germ-free mice feature significant changes in the structure of their intestine specifically: increased crypt lengths, increased cecum size (due to accumulation of mucins and biliar acids), decreased cellularity of PPs and decreased numbers of ILFs[110]. Also the mesenteric lymph nodes of these animals are smaller and have a decreased number of cells[111]. All these features are reversible upon microbial exposure. A direct transcriptomic comparison of the ilea from germ-free mice and animals colonized with *Bacteroides thetaiotaomicron* revealed differences in the levels of expression of multiple genes involved in glycidic and lipidic metabolism[112]. Additionally cell subset alterations have been shown to occur in these conditions, such as decrease in CCR6-T-bet$^+$RORγt$^+$ILC or absence/decrease of Th17 cells, with an increase in FOXP3+ cells and a total reduction in the number of LP CD4 T-cells[109]. Importantly, germ-free mice are also more susceptible to infection by bacteria, viruses and fungi, as illustrated by the increased mortality in these mice on infection with *Listeria monocytogenes*, *Shigella flexneri* or *Salmonella thyphimurium*[113]. This increased susceptibility is partially abrogated when the animals are exposed to specific commensal bacteria pre-infection.

In spite of the gut mucosa pro-inflammatory environment, inflammation is tightly controlled in order to limit tissue damage, and allow a peaceful relationship with the microflora. Therefore, it is not surprising that mucosal environment promotes the induction of Tregs. CD103$^+$ DC, through their production of TGFβ and RA, mediate local induction of these cells. Induced Tregs exert their suppressive function via several mechanisms: expression of inhibitory cytokines (IL-10, TGFβ, IL-35), scavenging of essential survival cytokines, like IL-2, and altering DC maturation through the expression of CTLA-4 and LAG-3[114].

ILC3 have now been recognized as important regulators of mucosal inflammation, both directly and indirectly. They control gut flora, lowering the likelihood of pathogenic Th17 responses[103]. On other hand, they induce IgA B-cell responses, via their expression of BAFF[101], assisting with the containment of gut flora. Finally, their expression of MHC class II allows them to directly interact with CD4 T-cells. This interaction does not result in CD4 T-cell proliferation, but anergy, which might help limit the pro-inflammatory milieu of the gut[115].

The use of more accurate methods to study microflora has helped establish an association between several disease states, immune responses to commensal flora and the predominance of specific bacterial strains in the gut[1]. Nevertheless, it is still difficult to establish a causal relationship, particular in human studies, with the strongest evidence coming again from the study of animal models, particularly comparisons between germ-free animals and animals housed under specific pathogenic free conditions.

The first associations of gut commensal flora with disease were observed in the context of inflammatory bowel disease (IBD) and gastrointestinal cancer. IBD includes two distinct inflammatory diseases of the gastrointestinal tract: Ulcerous colitis and Crohn's disease. Although they differ phenotypically and in terms of genetic susceptibility, both are linked with increased mucosal TNF levels[116]. Additionally, in both cases, an increase in the number of Th17 cells in the mucosa is apparent[117]. Although multifactorial in nature, several studies have linked dysbiosis to the pathogenesis of these diseases, however the triggers of these chronic inflammatory processes are still unknown. Evidence for an important contribution of commensal flora to these diseases comes from data demonstrating the ability of antibiotics to induce clinical remission[118]. On the other hand, data from animal models have suggested that colorectal cancer was co-transmissible with gut microflora, irrespective of genetic background[119, 120]. Additionally, recent data indicates a loss of gut mucosa homeostasis as an early event in septic shock and acute pancreatitis[121], contributing to the multiple organ dysfunction observed in these clinical situations[122].

Importantly, there is evidence of altered microflora in the context of chronic inflammation, with specific bacterial strains in the commensal flora being linked with pathogenic responses outside the gut. Using mice models, gut residing segmental filamentous bacteria, known to induce mucosal Th17 responses, were associated with autoimmune arthritis[6] and the presence of microflora alone, to the development of experimental autoimmune encephalomyelitis through induction of Th1 and Th17 responses[123]. Several other associations of dysbiosis with chronic conditions have been demonstrated, namely with obesity [124] and chronic kidney disease[125, 126].

Importantly, in HIV-1 infection, discussed bellow, the gut mucosal disruption and associated dysbiosis has been considered a key event in disease progression towards AIDS, contributing to the state of hyper-immune activation that underlies HIV immunopathogenesis.

Overall, this evidence has led to the belief that modulation of gut flora through diet and/or probiotics could offer an alternative therapeutic approach in several chronic and acute inflammatory disorders[127]. Nevertheless, the final proof is still lacking and more human studies are necessary to address the beneficial effects of adding probiotics to the human diet[128].

In summary, gut mucosal homeostasis maintains a delicate equilibrium between tolerance, necessary for the digestion and absorption of nutrients, and inflammation, essential for the control of the commensal flora. To achieve this state, multiple cell subsets come into play, namely epithelial cells, DC, Th17, Th22, Treg and ILC3. However, the global picture of how mucosa immune cells interact with gut epithelial cells and commensal flora is still framed work in progress, and a more complete knowledge is necessary in order to generate novel therapeutic interventions for the treatment of human disease.

1.2. The gut mucosa and HIV pathogenesis

HIV-1 infection is associated with gut mucosa disruption during acute infection[129, 130], a process which persists during all disease stages[131], and is not completely recovered under antiretroviral treatment, as reviewed by Costiniuk et al[132]. In this regard, the study of HIV infection has called attention to the importance of an adequate interplay between the gut environment and the host in order to maintain health. As early as 1989 the direct impact of HIV-1 infection on the gastrointestinal tract was recognized as a major player in HIV-1 induced pathogenesis, independent of the presence of opportunistic infections[133]. Subsequently several studies have provided direct and indirect evidence for gut disruption as a driver of disease progression[134].

On the contrary, there is a lack of knowledge regarding the impact of HIV-2 infection on GALT, and its potential relevance to the more benign course that characterizes this infection.

The next chapter will briefly review the mechanisms by which HIV-1 disrupts mucosal integrity and its overall relevance for HIV immunopathogenesis. Additionally, it will discuss the pathogenesis of HIV-2 infection with particular focus on the role of hyper-immune activation in CD4 T-cell depletion, despite the absence of plasma viremia.

1.2.1. HIV is still a major killer: overview of the HIV/AIDS epidemic

HIV-1 virus was discovered in 1983 after an outbreak of opportunistic diseases in a cohort of men who had sex with men in the United States (http://www.cdc.gov/mmwr/preview/mmwrhtml/june_5.htm)[135].

According to the latest UNIAIDS report, HIV-1 infection has killed 36 (30-42) million people worldwide since the start of the epidemic, and despite the successful introduction of effective ART in 1996 it still represents a major worldwide health problem (Figure 3). In 2013, 35.3 (32.2-38.8) million people were living with HIV-1. Moreover, despite a significant decrease in the rate of new infections (33% less that in 2001) due to a combined program of prevention measures (sexual counseling, barrier contraception use, anti-discrimination policies, circumcision and the use of global antiretroviral treatment (ART) as prevention), there were still 2.3 (1.9-2.7) million new infections reported worldwide in 2013. In Portugal in 2014, 48,657 individuals were living with HIV (prevalence around 0.7% in adults), with 1,416 new infections reported in 2013, accordingly to the 2014 report from the Programa Nacional para a Infecção VIH/SIDA of Direcção Geral de Saúde.

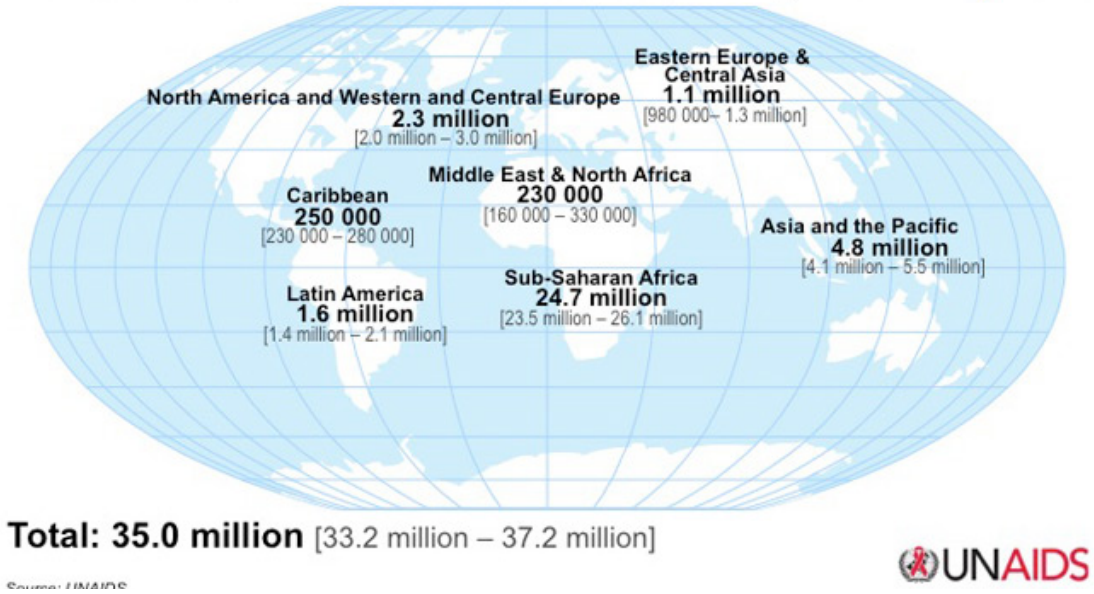

Figure 3. Global prevalence of HIV infection in 2013. *Adapted from UNAIDS report on the global epidemic 2013.*

Effective antiretroviral treatment (ART) has changed HIV-1 infection's natural history[136-139]. There has been a continuous reduction in the mortality of HIV-1 infected individuals due to AIDS events, as summarized in the 2013 UNAIDS report. Nevertheless, ART must be maintained life long, and these patients still feature an increased risk of death due to non-AIDS related morbidities, particularly cardiovascular disease[140].

In contrast, the infection by HIV-2, the second AIDS associated virus, is geographically confined to West Africa and countries related to this area, namely Portugal. Moreover it is associated with a reduced mortality and transmissibility[141], as described below.

1.2.2. The viruses behind the immunodeficiency syndrome: HIV-1 and HIV-2

HIV-1 and 2, along with HTLV-1 and HTLV-2, belongs to the lentivirus sub-family of the retrovirus family. The retroviruses are RNA viruses encoding a reverse transcriptase gene, which transcribes RNA into DNA that is then integrated into the host cell's DNA.

For both HIV-1 and HIV-2, the viral infectious particle consists of an envelope formed from host cell membrane, in which several viral proteins necessary for virus entry and fusion into other host cells are inserted, namely gp120 and gp41 for HIV-1 and gp105 and gp36 for HIV-2. This envelope surrounds the protein core or capsid, which contains the viral RNA that encodes the genes necessary for virus infection of host cells and replication. The core structure consists of several products of the Gag gene, and it also contains the vpr, protease, integrase and reverse transciptase proteins.

The virus genome has three structural genes: *gag*, *pol* and *env*, and several other accessory genes that interact with host cell proteins, facilitate proviral integration into the cell genome, and later, production of new viral particles. These are: *vpu* or *vpx* for HIV-1 or HIV-2 respectively, viral protease, viral integrase, Vif, Vpr and Nef (Figure 4).

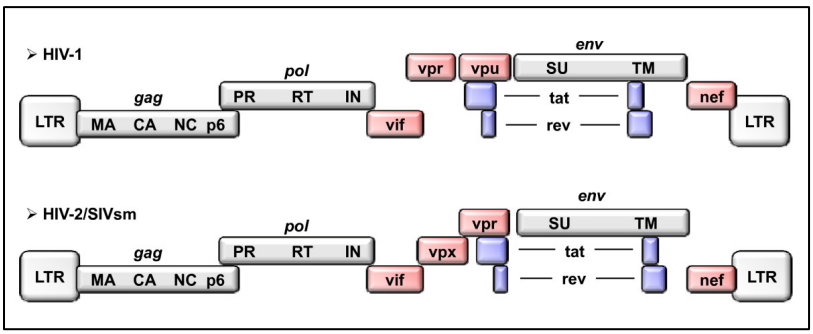

Figure 4. Schematic representation of HIV-1 and HIV-2 genomes. Grey boxes represent structural genes; blue boxes indicate regulatory genes; and pink boxes indicate accessory genes. *Adapted from Ayinde et al*[142].

The differences between HIV-1 and HIV-2 virus will be discussed in a subsequent section of this introduction.

To enter into a new host cell, HIV-1 membrane gp120 or HIV-2 gp105 proteins interact with the CD4 molecule at the cell surface. This interaction is fundamental for HIV entry into the cell, and leads to a conformational change in viral proteins that allows an interaction with a second cell co-receptor: CCR5 or CXCR4[143].

Upon recognition of CD4 and the specific co-receptor, fusion of the virus envelope and cell membrane occurs, mediated by a second viral glycoprotein (HIV-1 gp41 or HIV-2 gp36) and the viral core enters the cell cytoplasm of the target cell.

After entrance, the viral Matrix protein (MA or p17, a product of GAGmRNA), Nef and Vif mediate uncoating of the viral particle. The efficacy of this process depends upon cell activation, as immunossupressive agents like cyclosporine, an inhibitor of T-cell activation, impede this process[145]. The viral RNA is then retro-transcribed into DNA by the viral reverse transcriptase that partially uses the nucleotides available in the host cell cytoplasm. Although this process is restricted by APOBEC3G, which cause hypermutation in the viral genome, the virus counteracts through the inhibition of this restriction factor by Vif.

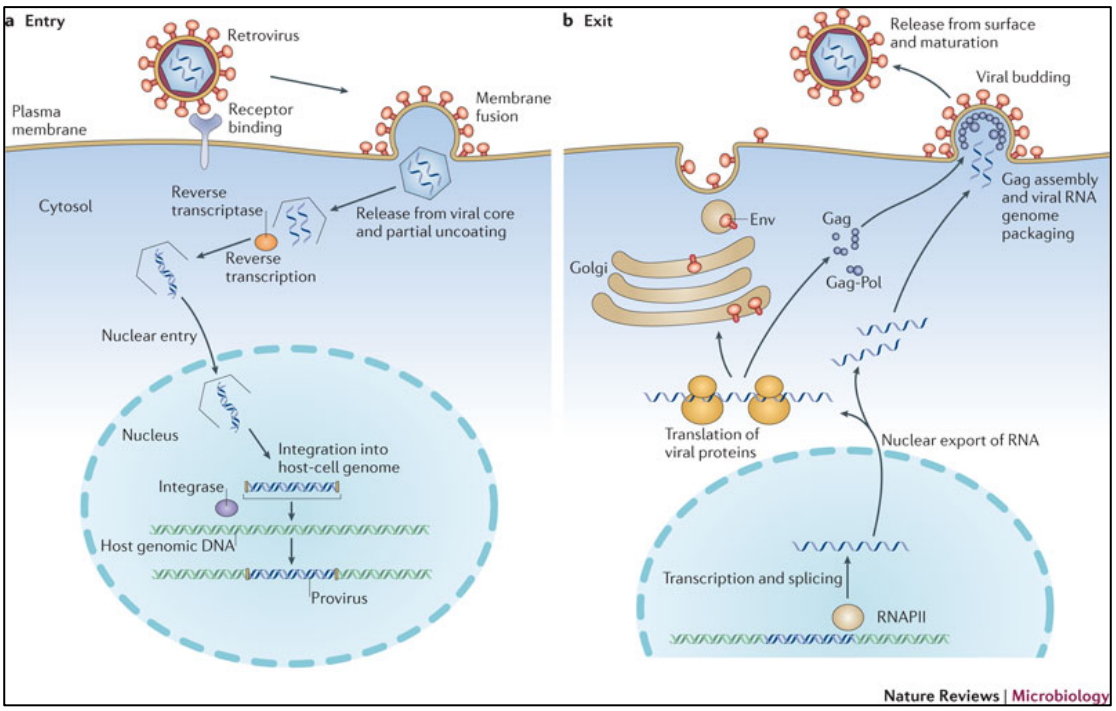

Figure 5. Illustration of the different events in the life cycle of retroviruses. A. Viral entry into cells involves the following steps: binding to a specific receptor on the cell surface; membrane fusion either at the plasma membrane or from endosomes (not shown); release of the viral core and partial uncoating; reverse transcription; transit through the cytoplasm and nuclear entry; and integration into cellular DNA to give a provirus. B. Viral exit involves the following steps: transcription by RNA polymerase II (RNAPII); splicing and nuclear export of viral RNA; translation of viral proteins, Gag assembly and RNA packaging; budding through the cell membrane; and release from the cell surface and virus maturation. Adapted from Stoye et al[144].

After double stranded viral DNA is formed, the viral protein Vpr facilitates its transport into the nucleus, where it can directly integrate into cell DNA or persist in the nucleus in several forms (linear, 1-LTR circle and 2-LTR circle) if the conditions for DNA integration are not met. The integration of viral DNA into the host genome is not a random process, as it occurs mostly in areas under regulation by cell activation factors. The quantification of integrated viral DNA (proviral DNA) has been extensively used to estimate the number of infected cells and is used as a measure of viral reservoirs[146, 147].

Following successful DNA integration, the provirus remains quiescent. Its transcription occurs on activation of viral promoters that are under the control of cell activation factors, specifically the NFκB pathway[148, 149]. This results in viral transcription upon T-cell activation, which can be directly induced by the presence of

virus, creating the perfect viral cycle. Viral mRNA is transcribed using cell machinery, mostly RNA polymerase II. Virus transcription efficiency is augmented by the viral protein Tat[150]. First, multiple-spliced forms of viral mRNA are produced and exported from the nucleus, and subsequently generating the accessory proteins Tat, Nef and Rev. The viral Rev protein facilitates the nuclear export of non-spliced viral transcripts. These can then either be translated into the structural proteins (Pol, Gag and Env) or remain untranslated and provide the viral genomic RNA. These structural viral proteins are subsequently cleaved by the virally-encoded protease generating the viral structural proteins and enzymes. During this process, and to prevent CD4 molecules scavenging the newly formed gp120, there is down-regulation of this molecule by Nef, Vpu and Env proteins. The envelope proteins then migrate to the host cell membrane, together with Gag proteins, followed by the several components that form a viral particle and an immature virion is released, completing the viral cycle.

1.2.3. HIV-1 natural history is linked to chronic hyper-immune activation

The absolute number and relative percentage of CD4 T-cells, together with viremia, are still considered the best currently available surrogate markers to classify and follow HIV-1 infected individuals. With respect to CD4 T-cell numbers, HIV-1 infection is classified by the Center of Disease Control (CDC) into three groups: 1) more than 500 cells/μL, 2) between 200 and 499 cells/μL and 3) less than 200 cells/μL, with an addittional A, B, C classification according to the presence of clinical evidence of immunodeficiency (A - asymptomatic, persistent generalized lymphadenopathy or acute serconversion illness; B – HIV-related conditions not classified in A or C; C - AIDS defining illness). Nevertheless, it is also clear that patients within the different CDC classes behave differently, and other markers are needed to better define immunocompetence and immunodeficiency in these individuals.

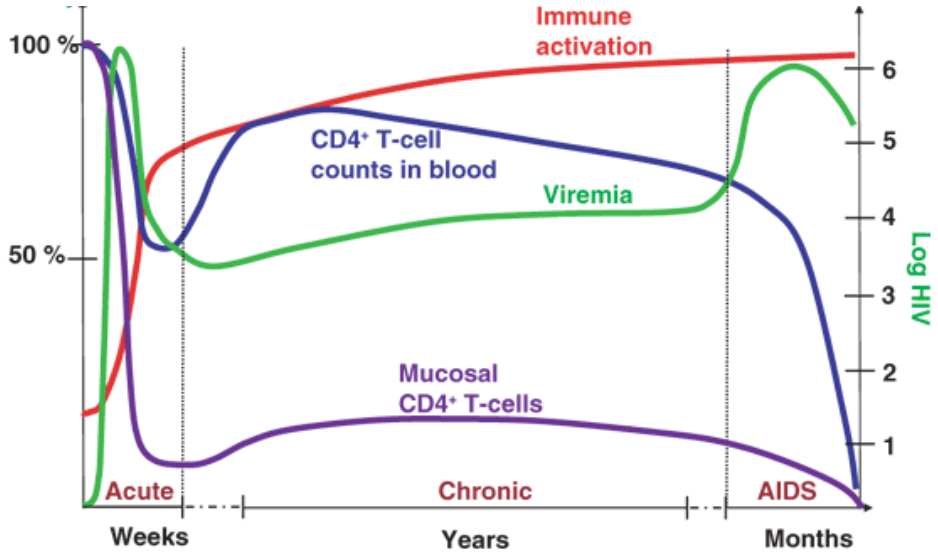

Figure 6. Schematic representation of HIV-1 natural history. The changes in mucosal and blood CD4+ T-cell numbers and in viremia level over the course of HIV infection are shown schematically in relation to changes in the relative level of immune activation. *Adapted from Grossman et al.*[151].

Acute HIV-1 infection is characterized by a rapid depletion of CD4 T-cells and a peak in plasma viremia (Figure 6). This period, although asymptomatic in more than 50% of patients, can be characterized by a mononucleosis-like syndrome, with fever, lymphadenopathy and organomegaly[152]. During this initial stage, anti-viral immune immune responses are induced in an effort to control viral replication. Cytotoxic CD8 T-cell (CTL) responses are one of the most important controllers of viral replication, and have been correlated with disease progression. CTL kill infected cells by direct cell contact or control viral replication through the release of IFNα or chemokines that bind the CCR5 co-receptor (MIP1α, MIP1β and RANTES).

Interestingly, the study of long-term non-progressors (LTNP), individuals followed for more than 10 years without evidence of CD4 T-cell decline, and of elite controllers, HIV-1 infected individuals that are able to control viremia, was fundamental to understanding the role of CTL as determinants of viral control[153, 154]. These patients have an over-representation of Human Leucocyte Antigen (HLA)-B*57[155, 156], which is associated with the induction of polyfunctional CD8 T-cell responses[157].

Acute infection is then followed by a long asymptomatic period, in which there is a progressive decline of CD4 T-cells and increase in viremia. During this period, there is destruction of immune niches important for T-cell homeostasis, localized in the thymus[158-161], lymph nodes[162] and GALT[134]. This stage is associated with a steady increase in the levels of chronic inflammation that fuels the infection of new cells, due to the persistent immune activated status of CD4 T-cells.

If left untreated, HIV-1 infected individuals progress to a state of severe immunodeficiency (AIDS) defined by less than 200 CD4 T-cells/mm^3, or/and the presence of AIDS defining illness. Such a degree of CD4 T-cell depletion in these patients is indicative of profound damage to the immune system.

HIV-1 disease progression, particularly CD4 T-cell depletion, has been linked to a variety of different mechanisms. There are several mechanisms that may lead to the HIV-mediated death of infected cells. For instance, upon integration into CD4 T-cells' DNA, the virus can directly induce cell death by activation of DNA-dependent protein kinase, a protein involved in the DNA damage response[163].

Nevertheless, the large proportion of cell loss is related to non-infected cells[164]. Human cohort studies have found that chronic immune activation, rather than viremia *per se,* is the major driver of CD4 T-cell depletion[165-167]. This might explain why in HIV disease states characterized by low viremia, such as HIV-2 infection, CD4 T-cell depletion still occurs[166]. Additionally, non-human primate studies have confirmed the important role of immune activation in driving disease progression. Both Sooty mangabeys (SM) and African green monkeys (AGM), natural hosts of Simian Immunodeficiency Virus $(SIV)_{sm}$ and SIV_{agm}, respectively, feature high levels of viremia, but low levels of immune activation, and have no evidence of disease progression[168]. Together with HIV disease progression, chronic inflammation has been linked to increased mortality by cardiovascular disease, an important cause of death in HIV infected individuals, even for those under ART[140, 165, 169].

Hyper-immune activation can be defined by a state of increased activation of immune system due to the continuous presence of an inflammatory insult,

irrespective of the causal mechanism. This state can be assessed using different indicators, from T-cell turnover, to the expression of cell-surface makers and plasma levels of inflammatory molecules, such as IL-1 and IL-6.

T-cell activation is associated with an up-regulation in the surface expression of HLA-DR and CD38. The frequency of CD4 and CD8 T-cells expressing these markers have been associated with HIV-1 disease progression, mortality and lack of immunological recovery under ART[170]. Additionally, monocyte activation, measured by the frequency of CD16 positive cells within CD14 bright monocytes, has also been shown to correlate with disease progression and mortality[171, 172].

Plasma biomarkers of inflammation, which are easier to measure, have been found to be associated with overall HIV-1-associated mortality, as well as cardiovascular disease. For example, the levels of soluble CD14 (sCD14), shed from activated monocytes, are correlated with overall HIV-1 survival[171]. In another large cohort study of American Veterans, HIV infection was associated with increased levels of IL-6, d-dimer and sCD14, after controlling for other major factors associated with inflammation known to be frequently overrepresented in the HIV population, like smoking, alcohol consumption or dyslipidemia[173].

Chronic immune activation is maintained via different mechanisms namely antiviral responses and homeostatic responses to CD4 T-cell lymphopenia among others, as recently reviewed by Hunt et al[165]. In fact, the homeostatic responses to CD4 T-cell lymphopenia per se may represent one of the major drivers of the hyper-immune activation[166]. Gut disruption, and the consequent increase in its permeability and microbial translocation is increasingly considered one of the most important factors.

1.2.4. Gut as the culprit for HIV-1 disease progression

The gut mucosa provides the perfect environment for HIV-1 viral replication, as it is a "theatre of war", where CD4 T-cells are continuously being activated by close contact with commensal flora, facilitating viral infection[174]. The understanding of HIV-induced disruption of gut homeostasis and its association with immune activation will hopefully lead to therapies complementary to ART.

However, despite the obvious relevance of gut disruption to HIV immunopathogenesis, the methods used to define the degree of gut disruption and the use of differing methodologies from study to study has hindered the translation of gut homeostasis assessment into clinical practice.

Gut disruption can be assessed by direct observation of gut mucosa via endoscopic methods and biopsy analysis. Although, this is the most accurate and validated method, it is usually impractical and is associated with possible complications. It may also lack sensitivity, being only able to provide data on gross structural changes in the gut organization.

Consequently, measurement of several molecules and other non-invasive approaches have been proposed as possible surrogate markers of gut disruption[175]. One of the first to be developed was the permeability test. In this assay non-digestible molecules of high and low molecular weight are ingested and their urinary excretion ratio is then measured. Different molecules have been used, with the assumptions that they are not metabolized in the gut or liver and that they are excreted in the urine in direct proportion to their absorption. Nevertheless, the data generated by these tests are not homogeneous or easily reproducible, raising the question of their utility as markers of gut disruption in clinical practice.

More recently, plasma markers of gut disruption have been tested in human studies, as indicators of the translocation of microbial products and/or gut epithelial lesion[176].

The presence of circulating microbial products can be assessed directly by measuring the levels of LPS (a protein constituent of gram-negative bacterial cell walls), D-Lactate (a by-product of bacterial metabolism)[177] or indirectly through the quantification of endogenous endotoxin-core antibody (EndoCAb)[178].

Furthermore, enterocyte damage can be indirectly assessed by the shedding into the circulation of proteins highly or uniquely expressed by these cells, specifically Intestinal Fatty-acid binding protein (I-FABP)[179, 180]. Likewise, zonulin serum levels have also been related with increased levels of gut permeability due to disruption of tight junctions[181].

In addition to molecules associated with the disruption and/or increased levels of permeability of the gut, the study of its integrity might also be complemented by the indirect study of mucosal inflammation levels. In this regard, the faecal levels of several molecules have been correlated with the presence of mucosal neutrophil infiltrates, especially calprotectin, also known as S100A8/A9[182]. Finally, systemic inflammation assessed by the expression of different activation markers at the single-cell level, as well as the levels of pro-inflammatory markers have been shown to correlate with several other markers of microbial translocation. Nevertheless, this is a very crude and unreliable way of assessing gut homeostasis.

In summary, there is no single marker that can be used to assess disruption of mucosal homeostasis and thus completely replace direct mucosal observation *via* endoscopic methods. It is with these limitations in mind that HIV-1's impact on GALT will be discussed.

1.2.4.1. HIV-1 induces profound changes in gut structure and function

Alterations in the architecture of the gut mucosa associated with HIV-1 infection, have been reported since the start of HIV pandemic. HIV-induced enteropathy is characterized by increased crypt length in the small bowel and decreased levels of brush border enzymatic activity[183]. In fact, the histopathological

changes associated with HIV-1 infection are very diverse, ranging from inflammatory infiltrates to ulcers, cryptitis, goblet cell hyperplasia and crypt deformation, a profile similar to that seen in IBD[184].

Despite these major changes in gut structure and function, HIV-associated gastrointestinal symptoms, specifically diarrhea, usually occur in the late phase of disease, at which point it is difficult to separate the direct effect of HIV from those of typical opportunistic infections such as CMV, *Isospora belli* or *Cryptosporidium*[185].

Morphological gut alterations are associated with modifications in the typical pattern of enterocyte gene expression, with up-regulation of genes involved in chemotaxis, IFN responses, and general inflammation, and down-regulation of genes involved in metabolic and digestive functions[186].

Together with these initial descriptions of HIV-induced enteropathy, in vitro models of mucosal cell infection by HIV supported a direct impact of HIV on gut epithelial cells[187]. HIV-1 can induce direct damage of these cells, opening the tight junctions, and thus contributing to the disruption of epithelial barrier function[188, 189].

Gut alterations were also found during SIV infection of *Rhesus Macaques* (RMs), a primate model of AIDS, namely: pathogenic changes in jejunal morphology, depressed digestive enzyme activity and abnormal mucosal T lymphocyte populations[190]. In contrast, the natural hosts of SIV, SM and AGM, preserve epithelial structure and present no evidence of leakage of microbial products into the mucosal lamina propria or the systemic circulation[191, 192], in spite of an acute depletion of mucosal CD4 T-cells[193].

HIV-1 is able to infect GALT cells in all areas of the gastrointestinal tract, from the gastric mucosa to the rectum[194]. The majority of the infected mucosal cells are activated CD4 T-cells, leading to their depletion from the lamina propria[131, 195]. This process is facilitated by the high levels of expression of HIV-1 co-receptors, CCR5 and CXCR4, on mucosal CD4 T-cells[52]. Additionally, HIV-1 infection is enhanced, as previously mentioned, by the continuous exposure to microbial products in the

mucosa lamina propria[196]. Cell populations in the GALT, other than CD4 T-cells, can be infected by HIV-1. Of interest, DC were recently shown to be attracted by HIV-1 envelope protein, and migrate between epithelial cells to sample HIV-1 virions[197].

Moreover, there are anecdotal reports of epithelial cell infection[198], although these data are inconsistent. It is plausible that viral detection in epithelial cells does not correspond to direct infection of these cells, but simply viral passage through the epithelium. Intestinal macrophages were also shown to be permissive to HIV infection in *in vitro* studies[199].

The relative abundance of cell targets in the gut results in higher levels of viral replication at this site, although the total number of cells infected, measured by proviral DNA, is comparable to that observed in peripheral blood[200].

Interestingly, as the gastrointestinal tract is composed of several niches, anti-viral responses probably differ between the different gut regions, leading to differential viral evolution between sites[201], thus contributing to the compartmentalization of HIV-1 infection and favouring the appearance of virus subspecies.

Gut mucosa viral replication, along with anti-viral responses, result in a rapid decline in LP CD4 T-cell count, from the very early phases of infection, in all areas of the gastrointestinal tract[202, 203]. This acute depletion of CD4 T-cells in the gut is much more striking than that observed in peripheral blood or in lymph nodes. The reasons for this remain elusive, although several hypotheses have been suggested. CCR5+ CD4 T-cells, the main target of the founder virus are highly abundant in the gut LP[52] providing a particularly favourable niche for virus replication[131]. Additionally, HIV-1 gp120 is able to engage the integrin α4β7, expressed by T-cells homing to the gut. On the other hand, this depletion is particularly marked in the LP, as compared to gut lymphoid aggregates[204], which might be due to a differential co-receptor expression by T-cells in the latter region.

In chronic phases of disease, in parallel to the bulk CD4 T-cell depletion, major imbalances in CD4 T-cell subsets in the lamina propria have been reported [205, 206]. As previously highlighted, IL-17 producing cells are fundamental in the gut to protect against bacterial and fungal infections, and these exist in a finely-balanced equilibrium with Tregs, that control immune response and promote tolerance to nutrients and gut flora[8].

IL-17 producing CD4 T-cells (Th17) are one of the major targets during HIV-1 infection[207]. The decrease in Th17 numbers is accompanied by a shift in their function, with a decrease in the ability to produce pro-inflammatory cytokines[205]. The particular targeting of this population by the virus might be related to its higher expression of the HIV co-receptor CCR5[207, 208], and decreased autocrine expression of the CCR5 ligand MIP-1β[208]. Consequently, there is an over-representation of Tregs in the gastrointestinal mucosa[209], which may potentially inhibit adequate HIV-specific T-cell responses. Additionally, high levels of IFNα production by plasmacytoid dendritic cells (pDC)[210] help maintain a chronic inflammatory environment, which in itself helps providing targets for viral replication. Th22 were recently shown to also be preferentially depleted in the acute and chronic phases of HIV-1 infection, and this was correlated with microbial translocation[189]. Reduction of mucosal IL-22 levels may indeed play a fundamental role in the pathogenesis of HIV-1 infection, given the importance of this cytokine in maintaining epithelial integrity. Notwithstanding this, there are several sources of IL-22 that could potentially compensate for the lack of IL-22 production by Th22, specifically ILC3, that are not direct viral targets, as in humans they lack CD4 expression[211].

The understanding of HIV-1 impact on gut homeostasis has been fueled by the study of pathogenic and non-pathogenic primate models of HIV infection as reviewed by Chahroudi et al [168]. Species-specific strains of SIV infect African primates in a non-pathogenic manner, and therefore these animals are viewed as their natural hosts, as in the case of SM or AGM, the main species studied in experimental settings. In contrast, infection of Asian macaques with a SIV_{mac}, a viral strain derived from SIV_{smm}, results in a pathogenic process similar to AIDS. The comparison between these two

models has clarified several aspects of HIV immunopathogenesis and ways in which to treat it.

Similarly to HIV-1 infection, acute SIV infection results in depletion of mucosal CD4 T-cells, both in pathogenic and non-pathogenic models. However, in the pathogenic RM model this is progressive, associated with loss of Th17 cells, and translates into microbial translocation and hyper immune activation, a process not observed in the non-pathogenic model[191]. In fact, the adequate control of immune activation in the latter, despite the characteristically high viremia, is likely the key to the absence of viral-associated pathogenicity and lack of disease progression. The preferential depletion of mucosal Th17 cells associated with pathogenic SIV infection, which is similar to that observed in HIV-1 infected humans, increases the risk of infections by bacteria such as *Salmonella*[212]. In addition, the imbalance between Th17 and Th1 cells in the gut has been established as a predictor of progression towards AIDS in this model[206]. Although mucosal Tregs were also depleted during acute SIV_{mac} infection, it was to a lesser extent than that of Th17, resulting, as for humans, in disease progression[213]. As previously mentioned, engagement of the α4β7 integrin by gp120 is one of the factors that potentially contributes to preferential depletion of mucosal CD4. This hypothesis was strengthen by data showing that treatment of RM with a monoclonal antibody that recognizes this integrin, prior to and during acute SIV_{mac} infection, resulted in a decrease in CD4 depletion[214]. Importantly, innate sources of IL-17, specifically IL-17 producing $Nkp44^{+}$cells, were also compromised due to increased IDO levels that limit tryptophan availability in the gut mucosa[215].

Understanding gut mucosal disruption by HIV-1 has also relied on the study of sub-groups of HIV-1 infected patients. In LTNP mucosal CD4 T-cells seem to be better maintained, particularly the Th17 subset[216]. These individuals also feature strong, polyfunctional mucosal HIV-specific CD4[217] and CD8 T-cell responses[218], which may limit viral replication at this site. These data from LTNP support the role of mucosal disruption to HIV disease progression. An apparent better-preservation of gut mucosal CD4 T-cells and of the balance between CD4 T-cell subsets was also described in elite controllers, defined as HIV-1 infected individuals with less than 75 RNA copies/mL in the absence of therapy[219].

1.2.4.1. Consequences of GALT disruption: Microbial products and chronic immune activation

The disruption of gut mucosa is not simply a key part of HIV-1 induced immunopathogenesis. Its consequences have a major impact on HIV disease progression, as has been shown in a landmark paper by Brenchley et al[220]. These alterations in gut mucosa are thought to increase permeability to microbial products, and thus contribute to the state of chronic hyper-immune activation that underlies CD4 T-cell depletion[167, 195].

The link between CD4 T-cell depletion, microbial translocation and immune activation has also been established in idiopathic CD4 T-cell lymphopenia[221], a syndrome in which there is no chronic viral infection to confound this association.

LPS, a gram-negative cell wall constituent, and bacterial DNA have been assessed in several HIV-1 infected cohorts, and their levels correlated with CD4[220], CD8[222] and monocyte activation, defined by high level expression of HLA-DR and CD38 on T-cells, and increased frequency of CD16 positive monocytes. In some cohorts, however, microbial products were only correlated with monocyte activation[223]. The reason for these inter-study discrepancies is not clear, and may be related to technical errors in LPS measurement, population size and/or the baseline characteristics of each cohort studied.

Importantly, LPS levels were shown not only to be associated to immune activation, but also be an independent predictor of disease progression irrespective of CD4 T-cell counts or viremia in ART naïve HIV-1 infected individuals[224].

Moreover, taking advantage of the pigtail macaque model of AIDS, where increased cardiovascular risk is recapitulated in the chronic phase of disease, treatment with sevelamer, an LPS-chelating agent, in the acute phase reduces immune activation, as well as rate of decrease of CD4[225].

However, microbial translocation in HIV-1 infection has to be viewed globally where failure of systemic mechanisms designed to counteract this process can occur. One such mechanism is the production of EndoCAb, an antibody that chelates LPS and is elevated during sepsis or IBD, limiting its deleterious effects. On the contrary, in chronic HIV-1 infected patients, there is an apparent decrease in EndoCAb production, which might be related to B-cell dysfunction[220].

Another possible mechanism to limit immune dysfunction associated with chronic elevated levels of LPS is the decreased production of pro-inflammatory cytokines by monocytes[226, 227], which has been confirmed in vitro using cells from chronically infected HIV-1 patients[220].

Despite an apparently better-preserved gut mucosal CD4 T-cell numbers and balance between CD4 T-cell subsets[219], elite controllers, in the absence of therapy, also feature increased levels of circulating LPS, which correlated with CD4 T-cell depletion and CD4 [228] and CD8 T-cell activation[229]. This data from elite controllers has raised doubts about the benign course of HIV disease in these patients, and the possible indication for ART, in spite of a low viremia and elevated CD4 T-cells.

Gut mucosa changes are also linked to changes in commensal flora[230, 231] and enteric virome[232], which by itself might re-enforce mucosal alterations. It has been recently shown that all subgroups of HIV-1 infected individuals feature a chronic dysbiosis that favours the outgrowth of Proteobacteria[233]. These microbes are capable of increasing mucosal levels of kynurine, which in turn can induce IDO production and the formation of tryptophan catabolites, thus contributing to the decrease of IL-17 producing cells[233].

Altogether, HIV-1 disrupts mucosal homeostasis contributing to the progression of disease towards AIDS. The control of this process in natural hosts, and partially in LTNP, strengthens the relevance of therapies designed to re-establish mucosa health.

1.2.4.2. Antiretroviral treatment is not the end for HIV-1 infection: role of persistent loss of gut mucosal homeostasis

Effective ART changed the natural history of HIV-1 infection from when it was first introduced in 1996[137-139]. Nowadays, HIV-1 infected individuals achieve an almost normal life-span if they comply with effective therapy[234], particularly if they start their treatment before the development of severe immunodeficiency[235]. Nevertheless, HIV-1 infected patients, as a whole, still feature an increased early mortality due to non-AIDS related events[236]. This increased mortality has been linked to persistent chronic immune activation, which in turn might contribute to the observed increased risk of cardiovascular disease[173]. Several other factors could also potentially contribute, particularly the very low but continuous levels of viral replication in tissues[237]. More recently, persistent disruption of GALT has been recognized as an important player in these processes[238].

Gut recovery has been investigated since ART became available. The first studies suggested that the restoration of peripheral blood CD4 T-cell counts, was paralleled by a recovery of mucosal CD4 T-cell numbers, a process that could occur as quickly as a week after starting treatment[239]. As more data became available, doubts were raised as to whether effective ART could completely re-establish mucosal homeostasis.

Several studies with variable sample size, patient age, time of follow-up and end points, generated different results regarding the degree of gut mucosal recovery achieved, as reviewed by Costiniuk, CT et al.[132].

On the one hand, after nine years of apparently effective antiretroviral treatment, there are still increased levels of markers of T-cell exhaustion in the mucosa, specifically increased expression of CTLA-4 and PD-1 on CD4 T-cells[240]. It was also observed that, despite increased mucosal CD4 T-cells and normalization of the Th17/Treg ratio, increased plasma levels of LPS persisted[241], as did decreased levels of EndoCAb, after long term suppressive ART[242]. On the other hand, some studies have shown a complete restoration of mucosal CD4 T-cells after long term ART started in patients with severe immunodeficiency[243]. Additionally, there is

evidence of a consistent decrease in microbial translocation markers, such as LPS or anti-flagellin antibodies in treated HIV-1 infected individuals[244], although this was not equally reproduced in all treatment groups. This raises the possibility that not all treatment regimens are similar in regard to re-establishment of the gut mucosa.

Overall, ART efficacy is classically determined by the absence of viremia. Nevertheless, there is evidence to show that plasma levels of antiretroviral drugs do not correlate with their tissue concentrations[245], which limit local control of viral replication. In fact, ART seems poorly able to reach the gut mucosa, which might explain the high levels of viral DNA found in the gut of ART-responsive patients, which correlated with persistent immune activation[237]. Indeed, one of the explanations for the lack of CD4 T-cell recovery is the persistence of an inflammatory environment[246] driven by low levels of viral replication. Inflammation, associated with IL-1β production, was shown to decrease stromal production of IL-7[247], as well as decrease responsiveness of CD4 T-cells to it[248]. Another argument favoring a role of IL-7 deprivation is data showing that IL-7 therapy, in combination with ART, can restore mucosal CD4 T-cell numbers and frequency, subsequently contributing to a decrease in mucosal inflammation[249].

Another hypothesis that could explain the apparent lack of recovery of gut homeostasis under ART is the irreversible deposition of collagen resulting from the persistent inflammation that existed prior to commencement of ART[250].

Therefore, it is critical to obtain a clearer understanding of the mechanisms that underlie gut disruption, in order to modulate microbial translocation and inhibit the chronic process of immune activation. In this regard, the study of particular groups of patients might provide additional clues to the pathogenesis of gut disruption in the context of chronic lentiviral infection. Thus, the study of gut mucosa in the context of HIV-2 infection, a naturally occurring model of attenuated HIV infection, might add significantly to the understanding of the role of immune activation and gut disruption in HIV immunopathogenesis.

1.2.5 HIV-2 infection: a naturally occurring disease that helps deciphering HIV immunopathogenesis

In 1986, HIV-2 was identified in the blood of patients from Guine-Bissau admitted to a portuguese Infectious Disease Department[251], after an initial report of a different lentivirus being detected in a group of Senegalese patients[252]. In the next year, the first description of 30 HIV-2 infected individuals, that apparently featured an equally rapid progression to AIDS and death as HIV-1 infected individuals[253]. However, as more HIV-2 infected patients were studied[254], it was quickly realized that HIV-2 infection was not associated with the same natural history[255-258].

HIV-2 infection is associated with a low mortality and low transmissibility. In fact, in West Africa, HIV-2 infection does not translate into decreased survival, although the relatively lower life expectancy should be taken in consideration[259]. Nevertheless, HIV-2 patients do eventually progress to AIDS featuring a clinical picture similar to HIV-1 infected individuals at this stage of disease[260, 261]. The reasons for this slower progression are still not clear, and do not seem to be the result of a longer time of co-evolution between the SIV, from which HIV-2 originates, and primates[262].

HIV-2 resulted from a zoonotic transmission of SIV, a primate lentivirus whose natural host primates are SM. It has been estimated that its transmission to humans occurred in the fifth decade of the 20th century[263]. The virus originating from this transmission event shares 60% nucleotide homology in the pol and gag genes with HIV-1, but only 30-40% in the other genes[264]. Regarding its structure and overall gene function, HIV-2 is very similar to HIV-1. In fact, HIV-2 uses the same primary receptor, CD4, and the co-receptors, CCR5 and CXCR4, as HIV-1, and thus targets the same cell populations. Although it can be more promiscuous in its co-receptor usage[265], using, in vitro at least, CXCR6, CCR2, CCR1[266], this has not been correlated with increased pathogenicity[267].

There is also evidence that it is able to establish a disseminated infection as assessed by the amount of proviral DNA adjusted for the CD4 T-cell count, or lymph

node proviral DNA [268, 269], although it might take longer to occur in the acute phase of infection, since HIV-2 seroconversion was never described, suggesting a slower progression of disease course right from the beginning of the infection.

As is the case for other lentiviruses, it is restricted by several host restriction factors. In fact, TRIM5α, a host restriction factor highly conserved among primates, is known to better restrict HIV-2 virus in comparison to HIV-1[270]. Nevertheless, TRIM5α molecules isolated from viremic HIV-2 patients are as efficient as those isolated from aviremic HIV-2 individuals in restricting the HIV-2 replicative cycle.

HIV-2 does not have a *vpu* gene, but instead contains *vpx,* which targets another important host cell restriction factor: SAMHD1. This factor depletes the pool of deoxynucleoside triphosphates, limiting reverse transcription, that is absolutely required for viral DNA synthesis[271]. In fact, the presence of Vpx relieves the inhibition of HIV-1 infection of macrophages in vitro[272]. It has been speculated that a, as yet unproven, more efficient HIV-2 infection of macrophages would lead to a better induction of innate immune responses. Of interest, a study comparing the efficiency of Vpx, from a small number of aviremic and viremic HIV-2 infected individuals, in antagonizing SAMHD-1 did not find significant differences[273]. Thus, the real importance of this finding for the understanding of HIV pathogenesis it is still unclear, and it remains the subject of active investigation.

HIV-2 infection is viewed as a naturally occurring form of attenuated HIV infection that may ultimately lead to the same degree of immunodeficiency as HIV-1, but over a much longer period of time. It has been estimated that HIV-2 infected individuals lose approximately 9 CD4 T-cells/μL per year, as compared to the 49 CD4 T-cells/μL lost on average by HIV-1 infected individuals[274]. Moreover, HIV-2 infection is not typically associated with detectable plasma viremia[166, 274], although when it is present it may result in a worse prognosis[275]. These features are probably associated with the low mortality attributed to this infection[259]. In fact, it was recently claimed that previous HIV-2 infection delays progression towards AIDS of individuals subsequently infected with HIV-1[276]. Additionally, and as a direct consequence of the low viremia, HIV-2 infection also features a low rate of transmission, both

longitudinal and vertical[277, 278]. This has resulted in a localized epidemic[141], mainly restricted to West Africa, where the first infection was thought to have occurred[258]. Nowadays, the West African nations with the highest prevalence of HIV-2 are Guinea-Bissau, and Gambia (UNAIDS global report 2013). Portugal, due to its historical connection with this area of the world, has the highest prevalence of HIV-2 infection in non-African countries, representing around 3% of all reported HIV infections[279].

The study of this natural model of attenuated disease has been used to address HIV immunopathogenesis, and has helped to clarify important mechanisms involved in this process[167, 280]. It relies on the study of several cohorts mainly established in West Africa, Portugal and France. Significantly, the methods used to quantify HIV-2 viremia are very heterogenous[281], and this has led to discrepant results related with viremia levels in HIV-2 infection. Of note, some studies do implicate viremia in CD4 T-cell loss[275] and immune activation[282, 283], while most of the data relate the slow CD4 T-cell loss preferentially with immune activation and less with viremia[166].

There is still no definitive explanation for the low levels of viremia observed in HIV-2 infection nor the host's apparently better ability to control the infection. It is even more puzzling since both HIV infections feature the same level of proviral DNA, when individuals are matched for the number of CD4 T-cells, meaning that HIV-2 is able to establish a systemic infection and viral reservoirs[269]. Evidence of ongoing viral replication regardless of viremia status also exists[269]. Furthermore, in vitro studies have reported similar degrees of cytopathicity upon HIV-2 and HIV-1 infections[284]. There are paucity of data on lymphoid tissues from HIV-2 infected individuals..

One possibility would be the development of more efficacious innate, cellular or humoral responses against HIV-2. Previous studies have shown that the production of broad and potent neutralizing antibodies may be related with a more open domain of the HIV-2 envelope[285], and they have been associated with CD4 counts[286]. Nevertheless, other cohort studies have not found the same correlation between the presence of neutralizing antibodies and disease progression[287], suggesting that this cannot be the only explanation for a better disease outcome.

Another hypothesis for the better control of HIV-2 viral replication would be stronger HIV-specific CD4 T-cell responses. Polyfunctional Gag-specific CD4 T-cell responses with high levels of production of both IL-2 and IFNγ, as well as an high proliferative capacity has been reported in HIV-2 infected individuals [288-290]. In fact, bulk CD4 T-cells from HIV-2 infected individuals maintain a better ability to produce IL-2 than those from HIV-1 infected individuals, which might contribute to a longer survival of these cells in the context of HIV-2 infection[291]. However, a detailed analysis of IFNγ production in response to HIV-1 and HIV-2 Gag was unable to find any difference between the T-cell response to the two infections[292].

Additionally, HIV-2 infected patients are known to better preserve the homeostatic mechanisms necessary to respond to lymphopenia, which may account for the slow rate of CD4 decline. A better preservation of thymus function has been previously demonstrated[293], thus maintaining the capacity to replenish the pool of naïve CD4 T-cells in HIV-2 infection. Furthermore, IL-7 associated homeostatic responses to lymphopenia are better preserved in HIV-2 infected individuals in comparison to their HIV-1 infected counterparts[294]. Moreover, HIV-2 infected individuals also seem to be able to better replenish their CD4 memory pool[295]. On the other hand, the levels of IFNα, important for the overall levels of immune activation, are lower in HIV-2 infection[296], and this might be the result of alterations in the pDC maturation process induced by HIV-2, resulting in their acquisition of an antigen presenting cell phenotype rather than one of IFNα production[297].

Nevertheless, and in spite of the low to undetectable viremia, CD4 depletion in HIV-2 infected individuals does occur, as well as similar unbalances in CD4 cell subsets, namely increased Treg[298], that directly correlate with immune activation, as demonstrated in the context of HIV-1 infection[166]. However, the causes underlying the hyper-immune activation state, and the mechanisms involved in its control, might differ between the two infections. For example, it was recently reported that CD4 T-cells from HIV-2 infected individuals up-regulate PD-1 and PDL-1 in tight correlation with the degree of CD4 T-cells depletion and immune activation[299]. This might be an important mechanism in limiting immunopathology, safeguarding cells from over stimulation.

As previously mentioned, the factors that contribute to immune activation in the context of HIV-2 infection are poorly understood, and may or may not relate to gut disruption. Although HIV-2 infected AIDS patients studied in Africa were reported to have increased levels of LPS[300], another cohort of HIV-2 infected individuals, at an earlier disease stage, did not feature an increase in LPS levels. Importantly, in these individuals, LPS levels did not correlate with monocyte activation or impaired responsive of monocytes to LPS[301].

There are as yet no studies addressing gut homeostasis and disruption in the context of HIV-2 infection. Given the importance of these processes in driving chronic immune activation and consequently disease progression in HIV-1 infection, investigation of local gut homeostasis in HIV-2 infection is fundamental to understanding the slow disease progression characteristic of this infection, and also HIV immunopathogenesis *per se*.

In summary, HIV-1's natural history and associated morbidities under ART are directly linked to gut disruption, microbial translocation and chronic immune activation. Understanding the mechanisms required to maintain gut homeostasis, in the context of a chronic mucosal lentiviral infection, that is associated with slow disease progression, like HIV-2 infection, will help identify new therapeutic targets with implications not only for HIV/AIDS but also for human health in general.

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CHAPTER 2. AIMS AND WORK PLAN

Human health is known to rely on a preserved gut homeostasis, which is regulated by a constant communication between the immune system and the commensal flora, as described in the first chapter. In consequence, gut disruption is now thought to underlie several pathogenic states, and in particular HIV-1-associated immunopathogenesis. In fact, loss of intestinal integrity occurs throughout the course of HIV-1 infection, and ultimately contributes to progression towards AIDS. Despite the increased survival following the introduction of antiretroviral treatment in 1996, HIV-1 infected individuals still feature an increased risk of non-AIDS related morbidities. This has been related to the persistently increased levels of immune activation, which are believed to result, at least partially, from the leakiness of the gut, despite the apparent control of viral replication with antiretroviral therapy (ART).

In contrast to HIV-1, HIV-2 infection is associated with low to undetectable viremia and a longer survival in the absence of antiretroviral drugs. In spite of its more benign course, HIV-2 infection is also linked with hyper-activation of the immune system, which is directly associated with the degree of CD4 T-cell depletion. Nevertheless, the rate of the CD4 T-cell loss is much slower in HIV-2 than in HIV-1-infected individuals.

The overall objective of this work was to further investigate gut homeostasis in the context of HIV infection, by asking two specific questions:

1) Is IL-22 dependent gut homeostasis restored upon prolonged antiretroviral treatment of chronic HIV-1 infection?

IL-22 is mainly produced by CD4 T-cells and type 3 innate lymphoid cells (ILC3), and was recently shown to be a fundamental cytokine for the maintenance of intestinal epithelial integrity. We hypothesized that disruption of IL-22 homeostasis may contribute to the persistent loss of epithelial integrity in treated HIV-1 individuals.

These results are described in Chapter 3.1.

2) Is gut homeostasis maintained in HIV-2 infected individuals, possibly contributing to the more attenuated course of the disease?

HIV-2 infection is characterized by slow CD4 T-cell loss, which is directly associated with the degree of hyper immune activation. The degree of gut damage associated with HIV-2 infection and its possible contribution to the state of chronic immune activation, have never been investigated. We hypothesized that gut homeostasis, particularly that related to CD4 T-cell balance, is preserved in this setting, contributing to a slower disease course in HIV-2 infection.

These results are described in Chapter 3.2.

To accomplish these aims, we recruited three cohorts: ART-treated HIV-1 infected, HIV-2 infected and seronegatives individuals, all of whom had clinical indication to perform colonoscopy, mainly for colorectal cancer screening or mild gastrointestinal symptoms. The ethical obligation to include only individuals with clinical indication to perform colonoscopy favored the enrollment of individuals with more than 50 years of age, with all groups being matched for age.

In parallel, we studied longitudinally an HIV-2 positive individual infected in the first year of life and diagnosed at the age of 18 years due to a clinical picture resembling chronic inflammatory bowel disease. This remarkable clinical case with disruption of gut homeostasis at the time of diagnosis, and clinical indication for follow-up colonoscopic studies, allowed us to study, over time, gut integrity before and after introduction of antiretroviral treatment.

In order to address mucosal homeostasis we used a comprehensive analysis focused on the evaluation of CD4 T-cell subsets and ILC3, using flow cytometry and immunohistochemistry/immunofluorescence histology methods. We combined this with a detailed study of intestinal structure, collagen deposition and integrity of epithelial tight junctions, by assessing ZO-1 expression.

Due to the limited amount of available biological material, we assessed overall mucosa environment through gene expression. First, we measured genes relevant for

IL-22 and IL-17 production, namely IL-23 and IL-6. We also assessed pro- (IL-1β or IL-6) and anti-inflammatory molecules (IL-10) to further characterize gut milieu. Finally, we measured gene expression levels of molecules downstream of IL-22 signaling, namely relevant mucins and microbial peptides.

Since chronic mucosal viral replication might help explain possible changes in the gut mucosa, the study of HIV-2 infected individuals included the immunohistochemistry assessment of HIV-2 Gag expression.

Finally, we investigated, in all the cohorts, the possible relationship between mucosal disruption and systemic immune activation levels, as well as with imbalances in the main CD4 T-cell subsets in the peripheral blood.

In agreement with the Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented here were published or are currently being prepared for publication:

Susana M. Fernandes, Ana R. Pires, Cristina Ferreira, Russell B. Foxall, José Rino, Carla Santos, Luís Correia, José Poças, Henrique Veiga-Fernandes, Ana E. Sousa.
Enteric mucosa integrity in the presence of a preserved innate interleukin 22 compartment in HIV type 1 treated individuals.
Journal of Infectious Diseases. 2014; 210: 630-40.

Susana M. Fernandes, Ana R. Pires, Cristina Ferreira, Rita Tendeiro, Luís Correia, Sérgio E. Paulo, Rui M.M. Victorino, Ana E. Sousa.
Gut disruption in HIV-2 infection despite reduced viremia.
AIDS. 2014; 28:290-2.

Susana M. Fernandes, Ana R. Pires, Paula Matoso, Cristina Ferreira, Luís Correia, Emília Valadas, José Poças, Patrícia Pacheco, Ana E. Sousa, Russell B. Foxall. Mucosa CD4 T cells and gut integrity are preserved in chronic HIV-2 infection
Manuscript in preparation.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. The gut in long term treated HIV-1 infection

3.1.1. Enteric mucosa integrity in the presence of a preserved innate IL-22 compartment in HIV-1 treated individuals

Enteric mucosa integrity in the presence of a preserved innate IL-22 compartment in HIV-1 treated individuals

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Running Head: Mucosal IL-22 in HIV-1 infection

Key Words: Gut Associated Lymphoid Tissue; HIV/AIDS; Mucosa Reconstitution; IL-22; Antiretroviral Therapy.

Published:

Fernandes, S.M. et al. Enteric mucosa integrity in the presence of a preserved innate interleukin 22 compartment in HIV type 1 treated individuals. *Journal of Infectious Diseases*. August 2014; 210: 630-40.

Abstract

***Background**:* Interleukin (IL)-22 is emerging as a key cytokine for gut epithelial homeostasis and mucosal repair. Gut disruption is a hallmark of HIV infection. Here we investigated IL-22 production and gut mucosal integrity in HIV-1-infected individuals receiving long-term antiretroviral therapy (ART).

***Methods**:* Biopsies from 37 individuals performing colonoscopy primarily for cancer screening, 17 HIV-1-infected and 20 healthy age-matched controls, were assessed.

***Results**:* We found significant depletion of sigmoid IL-22-producing CD4 T-cells (Th22) even after prolonged ART, contrasting with the apparently normal compartments of regulatory and IL-17-producing, as well as total mucosal CD4 T-cells. Despite the preferential Th22 depletion, IL-22-production by innate lymphoid cells (ILC) was similar to that observed in seronegatives, and transcription of molecules relevant for IL-22 production was preserved (*AHR*, *IL23*, *IL23R*, *IL1B*, *IL6*, and *TGFB1*). Remarkably, transcripts of IL-22 target genes (*REG3G*, *DEFB4A*, *S100A9*, *MUC1*, *MUC13*) were unaltered, suggesting an adequate production of antimicrobial peptides and mucins. In agreement, enteric epithelial architecture was fully preserved.

***Conclusion**:* Despite the reduced Th22 subset, innate IL-22-mediated mechanisms, essential for sigmoid mucosa integrity, were fully operational in long-term treated HIV-1-infected individuals. Our data highlight IL-22-production by ILC as an important target for therapies aimed at facilitating human mucosal reconstitution.

Introduction

Gut mucosa, the body's largest interface with the external environment, provides the first microbial barrier. Furthermore, its interaction with the microbiome actively shapes the systemic immune system and determines human health[1]. Interleukin (IL)-22 has emerged as a key cytokine in these processes[2]. Signalling via the IL-22 receptor induces proliferative and anti-apoptotic molecules in gut epithelial cells[3], shown to be critical for epithelial homeostasis, particularly in the context of mucosal insults. Additionally, IL-22 signalling triggers epithelial production of antimicrobial peptides, which are relevant both for the containment of the gut microflora, and in response to infections[4].

Whilst the IL-22 receptor is essentially expressed by non-hematopoietic cells, specifically the gut epithelia, IL-22 itself is produced, under tight regulation, by hematopoietic cells[2]. Of note, high rates of epithelial proliferation due to deregulated IL-22 production have been associated with colon cancer[5]. In the context of epithelial lesions, shedding of microbial products leads to the secretion of IL-23 by dendritic cells[6], which, in conjunction with IL-1β and IL-6, induces IL-22 production. On the other hand, IL-25 secretion by the intact/repaired epithelium is known to inhibit this process[7].

The main IL-22 producers in the gut mucosa are CD4 T-cells (Th22)[8], and a subset of innate lymphoid cells (ILC), named ILC3[9]. It is currently unknown whether factors governing IL-22 production impact distinctly on the above two populations, or if these populations differentially contribute to the preservation of mucosal integrity[10].

ILC3 were initially described as participating in the development of gut associated lymphoid tissue (GALT)[11], and were later shown to be critical for the response to tissue injury in adult life in the gut, thymus and lymph nodes[12, 13]. In addition to their non-redundant contribution to the control of attaching-effacing enteric infections[14], ILC3 were recently reported to suppress CD4 T-cell activation in response to commensal bacteria[15], further emphasizing their fundamental role in intestinal homeostasis.

IL-22 is likely to be implicated in HIV/AIDS pathogenesis, given the profound disruption of gut mucosa typically observed throughout disease[16]. Acute HIV-1 infection leads to massive loss of mucosal CD4 T-cells, that persists into the chronic phase[17], and is inconsistently recovered upon antiretroviral therapy (ART)[18]. Loss of epithelial integrity and high levels of circulating microbial products have been reported even in treated patients, and are considered important contributors to HIV-associated pan-immune activation that underlies HIV/AIDS pathogenesis[19-21]. The heightened activation state of the immune system is also associated with increased risk of non-infectious events in ART-treated individuals.

Most previous studies of the gut of HIV-1-infected patients focused on the loss of IL-17-producing cells[22]. However, given the overlap between the mucosal populations that produce IL-22 and/or IL-17[10], it is possible that some of the reported disturbances were due to IL-22. Interestingly, although IL-22 was initially described as a Th17 cytokine, there are several factors supporting a unique identity for IL-22-producing cells[8, 10]. Production of these two cytokines is linked to expression of the transcriptional factor retinoic acid orphan receptor (ROR)γt, and homing receptor CCR6[23]. However, aryl hydrocarbon receptor (AhR) ligands have a preferential impact on IL-22 production[24]. Additionally, TGFβ induces IL-17 secretion, whilst inhibiting IL-22 production, via c-Maf induction in CD4 T-cells[25]. Nevertheless, concomitant production of IL-17 may balance positive and negative impacts of IL-22, as suggested in inflammatory bowel disease[26].

The limited data available suggest that mucosal IL-22 production is reduced in chronic HIV-1[27] and Simian Immunodefiency Virus (SIV) infections[28]. However, mucosal distribution of enteric IL-22-producing cell populations in humans is still ill-defined. Furthermore, their relationship with the expression of genes involved in epithelial structure homeostasis is yet to be determined.

We performed a comprehensive study of Th22 and ILC3 cells in the sigmoid mucosa of long-term treated HIV-1-infected individuals, with effective control of viremia and immune reconstitution. We found histological evidence of mucosal integrity, despite a significant depletion of IL-22-producing CD4 T-cell as compared to

seronegatives. Strikingly, our study revealed preserved innate lymphoid cells and normal mucosal IL-22-induced molecules. Thus, our data indicate a dissociation of the mechanisms that maintain Th22 and ILC3 in the human gut, highlighting ILC3 as potentially important therapeutic targets in achieving mucosal integrity in HIV.

Methods

Studied cohorts

The study enrolled 37 individuals with clinical indication to perform colonoscopy (35/37 colon cancer screening) that were found to be cancer free: 17 HIV-1-infected under ART for a median of 8 years with evidence of effective virological and immunological response, and 20 seronegatives (Table 1). All individuals gave written informed consent for blood sample and gut biopsy collection and processing. Study conducted under approval of the Ethical Boards of the Faculty of Medicine of University of Lisbon, Hospitals Santa Maria and São Bernardo.

Cell isolation and culture

Twelve sigmoid biopsies per individual were collected from macroscopically normal mucosa, and processed immediately. 7-10 biopsies were digested with collagenase B (10mg/mL, Roche, Penzberg, Germany), at 37ºC, and then mechanically macerated. Lymphocytes were separated by Percoll gradient, and cultured for 16 hours at 37ºC in complete medium[29]. Cytokine production was assessed after 4-hour stimulation with phorbol myristate acetate (PMA), plus ionomycin, as previously described[29]. Blood was collected in parallel from 10 HIV-1-infected and 9 control individuals and peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Hypaque centrifugation gradient.

Flow cytometry

Cells were surface and intracellularly stained (Foxp3 Staining Buffer Set, Ebioscience), as previously described[30], using a panel of antibodies (see Supplementary Table 1), and acquired on Fortessa flow cytometer (BD Biosciences). Analysis was performed with FlowJo (version 9.3.1, Tree Star, Inc, Ashland, OR), after exclusion of dead cells (Live/Dead-LD, Life Technologies, Carlsbad, CA), within gated

populations containing ≥150 events (see Supplementary Figure 1). Circulating lymphocyte populations were characterized using whole blood staining, and analysed for activation markers as previously described[31, 32]. The following antibody combinations were used to identify mucosal ILC (IL-17/IL-22/cKIT/IFN-γ/NKp44/LD/CD45/CD3) and T-cell subsets (IL-17/IL-22/CD8/IFN-γ/CD4/LD/FOXP3/CD3). Cytokine production by PBMC was quantified using CCR7/IL-22/IL-17/IFN-γ/CD8/CD45RO/IL-2/CD3 within memory-effector T-cells. Unstimulated cells were used to define cytokine cut-offs.

Immunohistochemistry staining

One sigmoid and one ileum biopsy were embedded in paraffin after preservation in 4% formaldehyde. 3µm sections were stained with antibodies (see Supplementary Table 1), after paraffin removal with xylene, rehydration with alcohol, and antigen retrieval by heat. Cells were counted manually in ten images (400x) acquired with a Leica DM2500 brightfield microscope, and lamina propria area assessed using ImageJ 1.47i (NIH, USA), in blinded samples by two researchers. Haematoxylin and eosin (H&E), Masson's Trichrome and Periodic Acid Shiff – Alcian blue (PAS-AB) stainings were concurrently performed, and blindly evaluated by a trained pathologist. Samples were scored according to inflammatory alterations, type of infiltrate, presence or absence of fibrosis, and number of mitotic figures at the crypt base with scores ranging from 0 to 3.

Immunofluorescence staining

One sigmoid and one ileum biopsy were frozen in OCT (VWR, Radnor, PA) after six-hour preservation in 4% paraformaldehyde, followed by 24 hours in 18% sacarosis to preserve biopsy structure. 3µm tissue sections were stained by sequential antibody incubations (see Supplementary Table 1), after protein blocking. DAPI was used for nuclear counterstaining. Images were acquired with Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a Plan-Apochromat 20x/0.8 objective, surface area measured with ImageJ 1.47i, and positive cells counted using in-house software, written in MATLAB (Mathworks, Natick, MA), allowing automatic generation of individual report images for cell-counting verification (see Figure 1).

RNA Extraction and analysis

One sigmoid and one ileal biopsy were stored in RLT-buffer (Qiagen, Valencia, CA) immediately post-collection. RNA was extracted using Allprep RNA/DNA mini kit (Qiagen), and 250ng used to synthesize cDNA (SuperScript III, Life Technologies). Expression levels of *IL22*, *IL17A*, *IL22RA1*, *IL23*, *IL23R*, *IL25*, *TGFB1*, *IL1B*, *IL6*, *MUC1*, *MUC13*, *S100A9*, *REG3G*, *DEFB4A*, *AHR* and *IDO1* were measured after pre-amplification with TaqMan Preamp Master Mix, using TaqMan gene expression assays with an Applied Biosystems 7500 Fast Real-Time PCR System (all from Life Technologies). Results are expressed as delta CT normalized to the medium CT levels of *GAPDH* and *r18S*. When gene expression was undetectable, a value 40 CT was used for the purposes of statistical analysis.

Statistical Analysis

Results are expressed as medians and interquartile range. Comparisons between two groups were made using Mann-Whitney U test, using Graph Prism version 5.0 (GraphPad Software, San Diego, CA). Non-adjusted regression analysis or Spearman correlation was used to identify associations between several parameters and multivariate linear regression analysis used to adjust results for age or sex using STATA 12.1 (StataCorp, College Station, Texas, USA). Analysis and presentation of distributions of IL-22 producing cells was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov. Comparison of cytokine-producing subset distributions was performed using Wilcoxon Signed Rank test and partial permutation test[33]. *P* values below 0.05 were considered significant.

Results

IL-22-producing cells in the sigmoid mucosa

Mucosal IL-22 production was evaluated in sigmoid biopsies from 17 long-term treated HIV-1-infected individuals with effective suppression of viremia and evidence of immunological reconstitution, and 20 seronegatives (Table 1).

Table 1. Cohort Characterization

	Control	HIV-1[a]
Number [male/female]	20 [7/13]	17 [13/4]
Age (y)	58 (49-63)	58 (52-60)
Length of follow-up (y)	NA	12 (8-12)
Length of ART[b] (y)	NA	8 (6-11)
Nadir CD4 T-cells/μL before ART	NA	339 (162-389)
Viremia (RNA copies/mL)	NA	< 40
CD4 T-cells/μL	619 (566-739)	624 (326-774)
CD8 T-cells/μL	375 (296-521)	884 (587-1197)[c]
% Naïve ($CD45RO^-CCR7^+$) within CD4 T-cells[d]	29.4 (23.2-33.4)	31.6 (26.9-43.8)
% Naïve ($CD45RO^-CCR7^+$) within CD8 T-cells[d]	16.1 (11.7-25.3)	15.3 (5.5-26.8)
% $HLA\text{-}DR^+CD38^+$ within memory CD4 T-cells[d]	7.5 (6.3-8.7)	10.8 (7.1-15.4)
% $HLA\text{-}DR^+CD38^+$ within memory CD8 T-cells[d]	21.1 (17.0-23.6)	29.3 (14.8-46.1)
% $CD16^+$ within $CD14^{bright}$ monocytes[d]	14.4 (12.8-14.7)	16.3 (11.9-28.2)
% $IL\text{-}22^+$ within memory CD4 T-cells[d]	1.5 (0.9-1.5)	1.5 (0.8-1.7)
% $IL\text{-}17^+$ within memory CD4 T-cells[d]	0.7 (0.6-1.5)	0.9 (0.6-1.9)
% $IFN\gamma^+$ within memory CD4 T-cells[d]	10.2 (6.8-38.8)	13.7 (9.2-18.4)

Data expressed as medians, interquartile range in brackets. Statistical analysis performed with Mann Whitney U test. NA: not applicable. [a]HIV-1-infected individuals were followed at the Departments of Infectious Diseases of the University Hospital de Santa Maria, Lisbon, and Hospital São Bernardo, Setúbal, Portugal. [b]Triple therapy in all patients (protease inhibitors in 8/17). [c]$P<.05$ to controls. [d]Peripheral blood analysis of 10 HIV-1-infected and 9 control individuals.

The total amount of IL-22 production in the sigmoid mucosa was quantified within hematopoietic cells defined as CD45+ (see Supplementary Figure 1).

We observed a significant depletion of IL-22-producing cells in the sigmoid mucosa of treated HIV-1 individuals as compared to age-matched controls, in the context of a preserved frequency of $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells (see Supplementary Figure 1). In agreement, the number of IL-22 positive cells, as determined by immunofluorescence, was significantly reduced in HIV-1 individuals as compared to controls (73cells/mm^2[43-170] versus 222cells/mm^2[152-297], P=0.003). Although,

the reduced number of ileum biopsies per subject precluded single-cell isolation, immunohistochemistry studies revealed similar numbers of IL-22$^+$ cells in the ileum of HIV-1 patients when compared to seronegatives (see Supplementary Figure 2). Our data are in line with previous studies demonstrating a distinct impact of HIV-1 infection of colon and ileum[34].

Overall, both the frequency and total numbers of IL-22-producing cells were significantly reduced in the sigmoid mucosa in HIV-1 infection even after long-term ART.

Mucosal Th22 cells

The immunofluorescence staining of IL-22 and CD3 showed a significant depletion of IL-22-producing T-cells in HIV-1+ patients as compared to seronegatives (Figure 1).

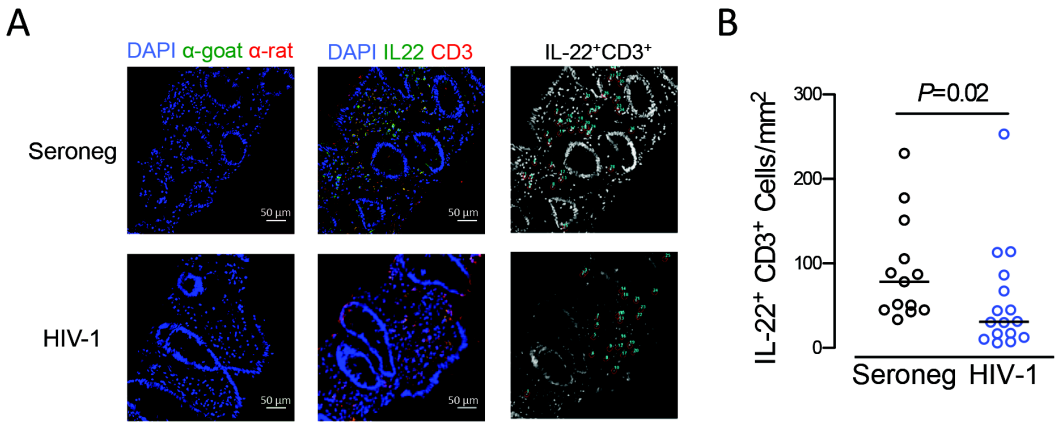

Figure 1. Depletion of IL-22-producing T-cells in the sigmoid mucosa of treated HIV-1 individuals. A) Representative IL-22 immunostaining in the sigmoid mucosa of HIV-1-infected and control individuals. White bars in figures represent 50µm. CD3 positive that express IL-22 were counted using a program developed inhouse. Briefly, single cell nuclei were identified, via DAPI, by thresholding and particle analysis, and dilated regions of interest (ROIs) based on a user-defined radius were defined from each nuclei, as the corresponding cellular areas for the other three channels. For each channel and ROI, a staining was considered positive if a minimum number of pixels (usually 5) were above a given threshold, and a particular combinatorial filter for cell counting was defined based on staining (e.g. positive staining in channels A and B but not in C or positive staining in channels B and C regardless of A). Cell numbers are shown in red. B) Number of IL-22$^+$CD3$^+$ cells by mm^2 in HIV-1 (n=16) and seronegative (n=13) cohorts. Each dot represents one individual and bars represent median in the graphs. Groups compared using Mann-Whitney U test. *P* values are shown.

CD4 T-cells were shown to be the main producers of IL-22 in the human gut[2]. Our analysis of cell suspensions confirmed that adaptive IL-22 production was mostly restricted to mucosal CD4 T-cells, although some subjects featured a few IL-22-producing CD8 T-cells (see Supplementary Figure 1, less than 2.5%, detectable in 5/15 HIV-1 and 4/13 controls).

We found that the frequency of IL-22-producing cells within CD4+ subset was significantly decreased in treated HIV-1+ individuals (Figures 2A and 2B), even when adjusting for total number of mucosal CD4 T-cells (R=-5.9; *P*=0.008).

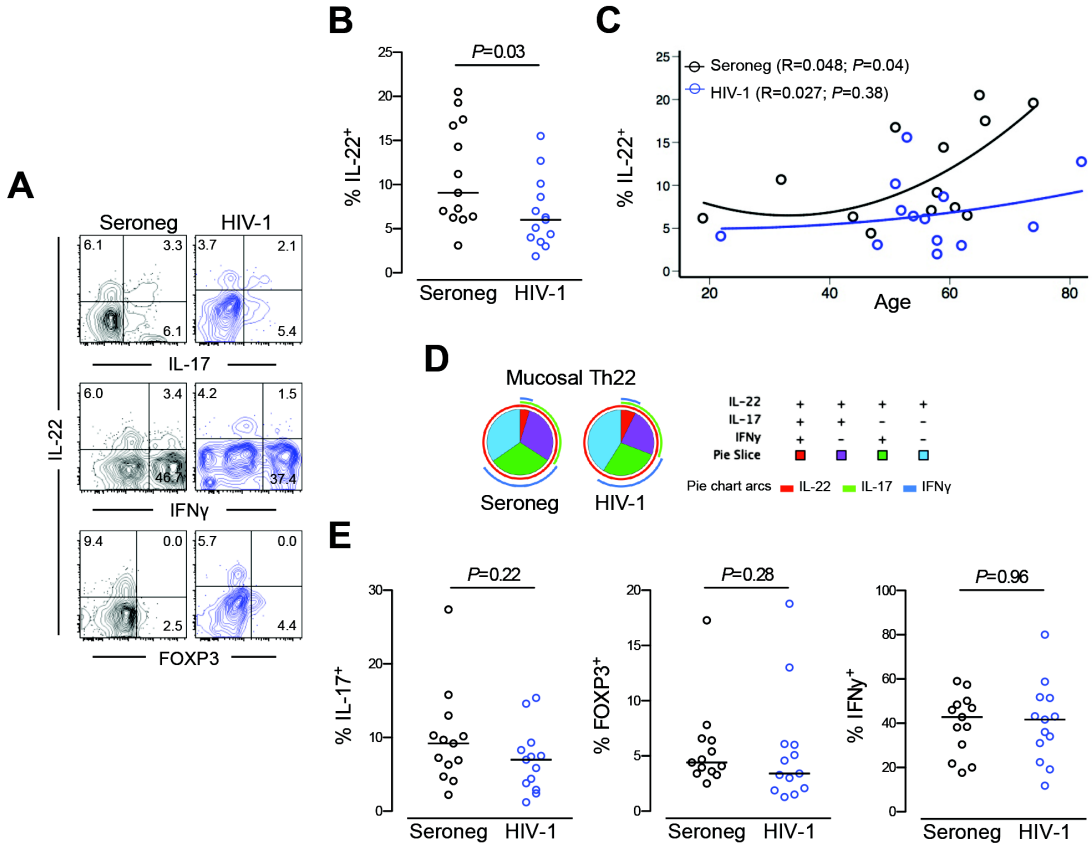

Figure 2. ***IL-22-producing CD4 T-cells in the sigmoid mucosa*** A) Representative dot plots of IL-22, IFN-γ, IL-17 and FOXP3 expression within gated mucosal CD4+ T-cells from HIV-1-infected and control individuals. Cytokine production was assessed upon PMA-ionomycin stimulation of cells isolated from biopsies of macroscopically normal mucosa. Numbers inside quadrants represent the frequency of positive cells. B) Frequency of IL-22+ within total CD4 T-cells in HIV-1 (n=13) and seronegative (n=13) cohorts, and its association with age using multiple linear regression analysis to calculate R coefficients, including a quadratic term for age, is shown in C). D) Distribution of IL-17 and IFN-γ production among total IL-22-producing CD4 T-cells in HIV-1 and seronegative cohorts, determined using SPICE software 5.0. E) IL-17+, FOXP3+ and IFN-γ+ cells within total CD4 T-cells in HIV-1 and control cohorts. Each dot represents an individual and bars represent median. Groups compared using Mann-Whitney U test and *P* values are shown.

Of note, our long-term treated HIV-1 cohort showed no significant reduction of CD4 T-cell counts in the sigmoid mucosa, though an expansion of CD8 T-cells was still observed (see Supplementary Figure 1).

The levels of mucosal Th22 were neither related to peripheral blood CD4 T-cell counts (R=-0.031, *P*=0.89, n=22), nor to frequency of circulating IL-22-producing CD4 T-cells (R=0.1, *P*=0.72, n=17), the latter being similar in HIV-1 and control cohorts (Table 1). Importantly, there was no association between mucosal Th22 frequency and nadir CD4 T-cell count before ART, or length of therapy.
Interestingly, aging was associated with a progressive increase in IL-22-producing cells within mucosal CD4 T-cells in healthy subjects (Figure 2C). This relationship was not observed in the HIV-1 cohort (Figure 2C), supporting a disturbed homeostasis of this subset in the sigmoid mucosa, even after long-term ART.

Nevertheless, the proportion of Th22 cells that concomitantly produce IL-17 and/or IFN-γ revealed no significant imbalances in HIV-1 patients as compared to healthy individuals (Figure 2D); with all IL-22-producing CD4 subsets under-represented in HIV-1-infected cohort (see Supplementary Figure 1).

Mucosal Th22 cells had significantly more co-expression of IL-17 and/or IFN-γ than the circulating counterparts in both cohorts (data not shown). Thus, despite the major Th22 depletion found in HIV-1 patients, the factors governing the balance of these Th22 sub-populations seemed to be operating as in controls.
The frequency of the other main CD4 T-cell subsets, namely IL-17$^+$, IFN-γ$^+$ and FOXP3$^+$, were similar in the two cohorts (Figure 2E), supporting a preferential depletion of Th22. Accordingly, immunohistochemistry revealed similar numbers in HIV-1 and seronegative cohorts of IL-17$^+$ cells (123cells/mm^2[60-202] , n=15, versus 82cells/mm^2[77-149], n=15; *P*=0.74) and FOXP3$^+$ cells (37cells/mm^2[26-87] , n=17, versus 54cells/mm^2[14-87] , n=17; *P*=0.89).

In conclusion, long-term treated HIV-1 individuals exhibited a significant depletion of Th22 cells in the sigmoid mucosa.

Levels of molecules known to regulate IL-22 production in the sigmoid mucosa

Next, we investigated possible alterations in the expression levels of molecules known to modulate IL-22 production in the gut. We found no alterations in the mRNA expression levels of *IL23*, *IL23R*, *IL6*, *IL1B*, *AHR*, *IL25*, *IDO1*, and *TGFB1* (Figure 3).

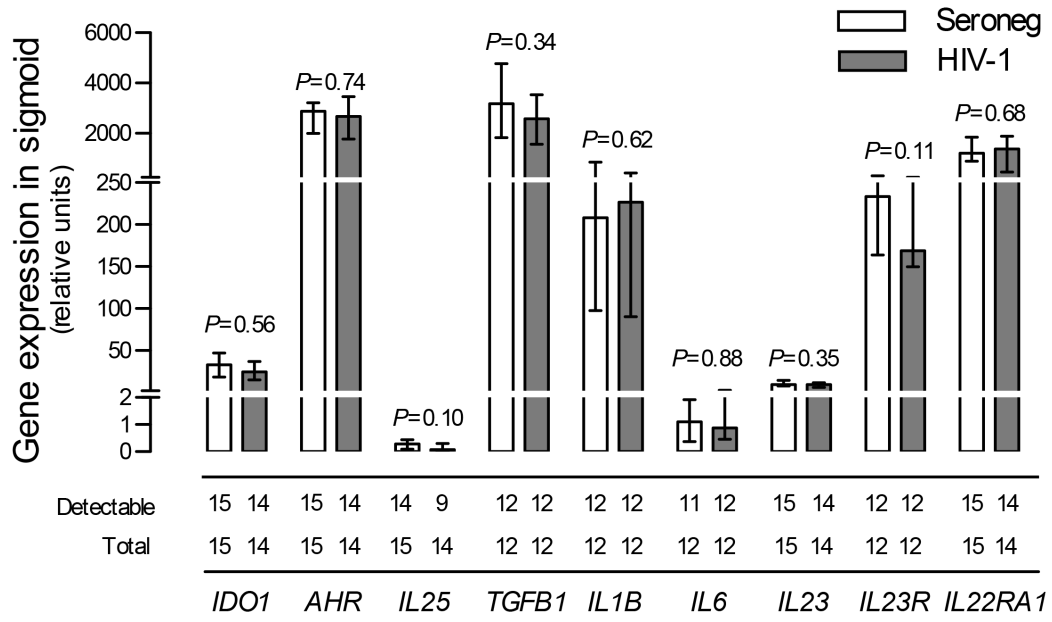

Figure 3. Levels of expression of genes known to modulate IL-22 production and/or function. *IL23, IL25, AHR, IDO1, IL6, IL1B, IL23R* and *IL22RA1* mRNA levels in total RNA extracted from sigmoid biopsies of HIV-1-infected and seronegative individuals. Numbers below indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*. Bars represent medians and interquartile range. Comparisons were made using Mann-Whitney U test; *P* values are shown,supporting the lack of any significant differences.

Moreover, the levels of these transcripts were not significantly associated with the frequency of IL-22-producing cells, either within total hematopoietic cells or CD4 T-cells ($P>0.3$). Similar findings were obtained in ileum biopsies (see Supplementary Figure 2).

Thus, the expression levels of the main regulators of mucosal IL-22 production were preserved in treated HIV-1+ individuals, suggesting they feature a normal IL-22 permissive environment.

IL-22-producing non-T-cells and ILC3 in the sigmoid mucosa

Next we investigated IL-22-production by non-T-cells, and found that it was comparable in the two cohorts (Figure 4A).

Notably, HIV-1-infected and control individuals featured a similar profile of concomitant production of IL-17 and/or IFN-γ by non-T-cells, with a marked prevalence of IL-22 single-producing cells in both cohorts.

Additionally, we performed a triple immunofluorescence staining for CD3, RORγt, and IL-22 in the sigmoid mucosa, and confirmed that the number of both $CD3^{-}RORγt^{+}$ cells, and $CD3^{-}RORγt^{+}IL\text{-}22^{+}$ cells were similar in the two cohorts (Figure 4B).

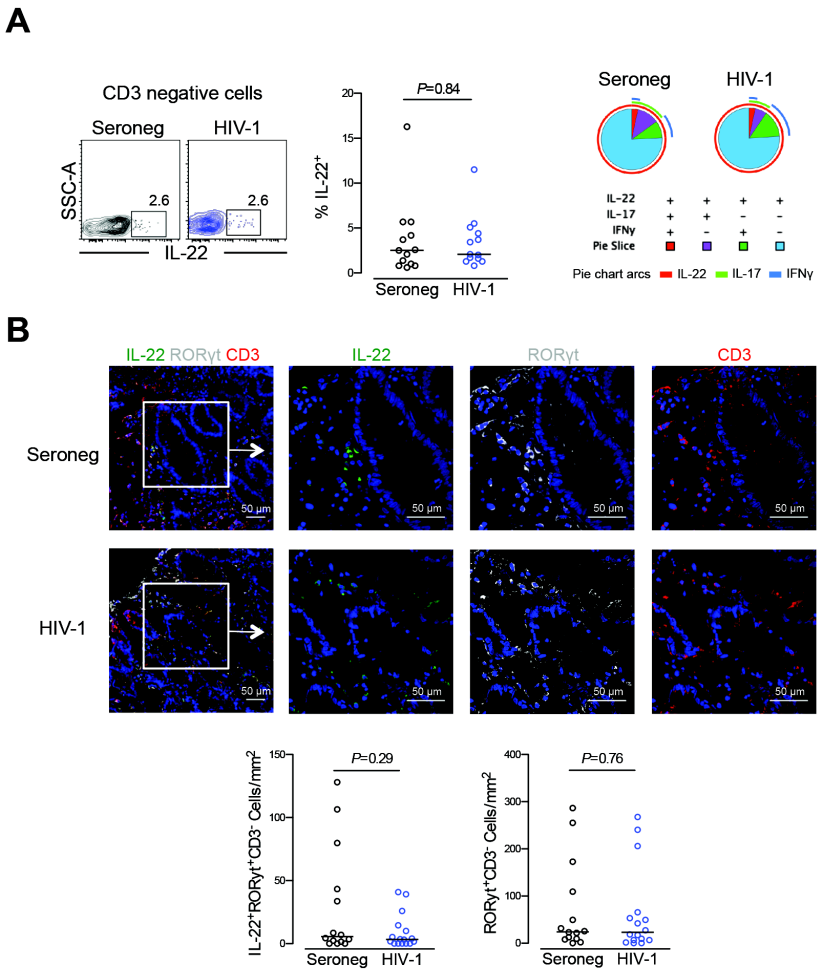

Figure 4. ***IL-22 production by non-T-cells in sigmoid mucosa.*** A) Flow cytometry analysis of IL-22 production within gated $CD3^{-}$ cells from HIV-1-infected and control subjects showing representative dot-plots with proportion of positive cells depicted inside boxes (left); frequency within HIV-1 (n=13) and control (n=13) cohorts (centre); and distribution of IL-17 and IFN-γ production among these cells in both cohorts, using SPICE software 5.0 (right). B) Immunofluorescence quantification of IL-22-producing cells in sigmoid biopsies of HIV-1-

infected and control individuals showing representative images of IL-22, RORγt and CD3 staining, with white bars indicating 50μm (top); and mucosal counts of RORγt^{+}CD3^{-} and IL-22^{+}RORγt^{+}CD3^{-} cells in HIV-1 (n=16) and control (n=14) cohorts (bottom). Each dot represents an individual, and bars indicate medians in the graphs. Analysis was performed using Mann-Whitney U test, and *P* values are shown.

The main source of innate IL-22 in the gut is a subset of innate lymphoid cells that express the natural cytotoxic receptor NKp44: ILC3. HIV-1-infected individuals showed preserved frequency of mucosal ILC3 cells, defined by the expression of cKIT and NKp44 within CD3 negative cells, as compared to seronegatives (Figure 5A). These cells expressed RORγt (Figure 5B), which is essential for their development and function[35].

Moreover, similarly to healthy, the mucosal ILC of HIV-1+ individuals also expressed high levels of the IL-7 and IL-2 receptor α-chains (CD127 and CD25, respectively, see Supplementary Figure 1)[36]. In agreement with previous data[37], we found that the ILC3 subset was the main producer of IL-22, in both healthy and HIV-1-infected individuals, with no skewing towards IFN-γ production (Figure 5B).

Of note, a multivariate regression analysis including HIV-status and gender as co-factors confirmed that gender neither biased our results regarding ILC frequency (*P*=0.42), nor IL-22-producing non-T-cells (*P*=0.49) or CD4 T-cells (*P*=0.18).

Hence, in contrast to Th22 depletion, treated HIV-1 patients featured a preserved frequency of innate IL-22-producing cells.

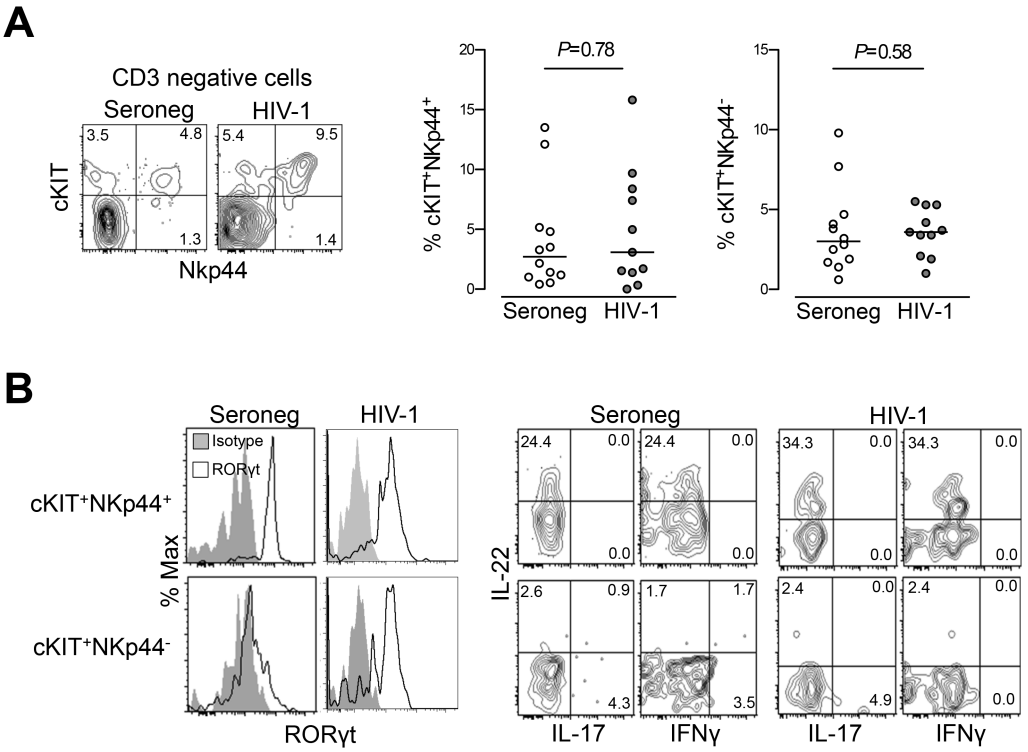

Figure 5. ***Innate lymphoid cells in the sigmoid mucosa.*** A) Representative dot plots of cKIT and NKp44 expression within gated CD3⁻ cells from HIV-1-infected and control individuals. Numbers inside quadrants represent frequency of positive cells. Graphs show the frequency of cKIT⁺NKp44⁻ and cKIT⁺NKp44⁺ within CD3⁻ cells from HIV-1 (n=11) and control (n=12) cohorts. Each dot represents an individual, and bars indicate medians in the graphs. Analysis was performed using Mann-Whitney U test; *P* values are shown. B) Representative analysis of RORγt expression, as well as IL-22, IL-17 and IFN-γ production within cKIT⁺NKp44⁻ and cKIT⁺NKp44⁺ gated CD3⁻ cells from the sigmoid mucosa of HIV-1-infected and control subjects.

Epithelial preservation in long-term treated HIV-1+ individuals.

Finally, we investigated the degree of sigmoid epithelial disruption in the context of marked Th22 depletion but preserved ILC3.

Initially, we quantified expression of IL-22 target genes, which are likely to help maintain epithelial integrity. We found that the genes for mucins (*MUC1* and *MUC13*) and antimicrobial peptides (*REG3G*, *S100A9* and *DEFB4A*) were similarly expressed in the HIV and control cohorts (Figure 6A).

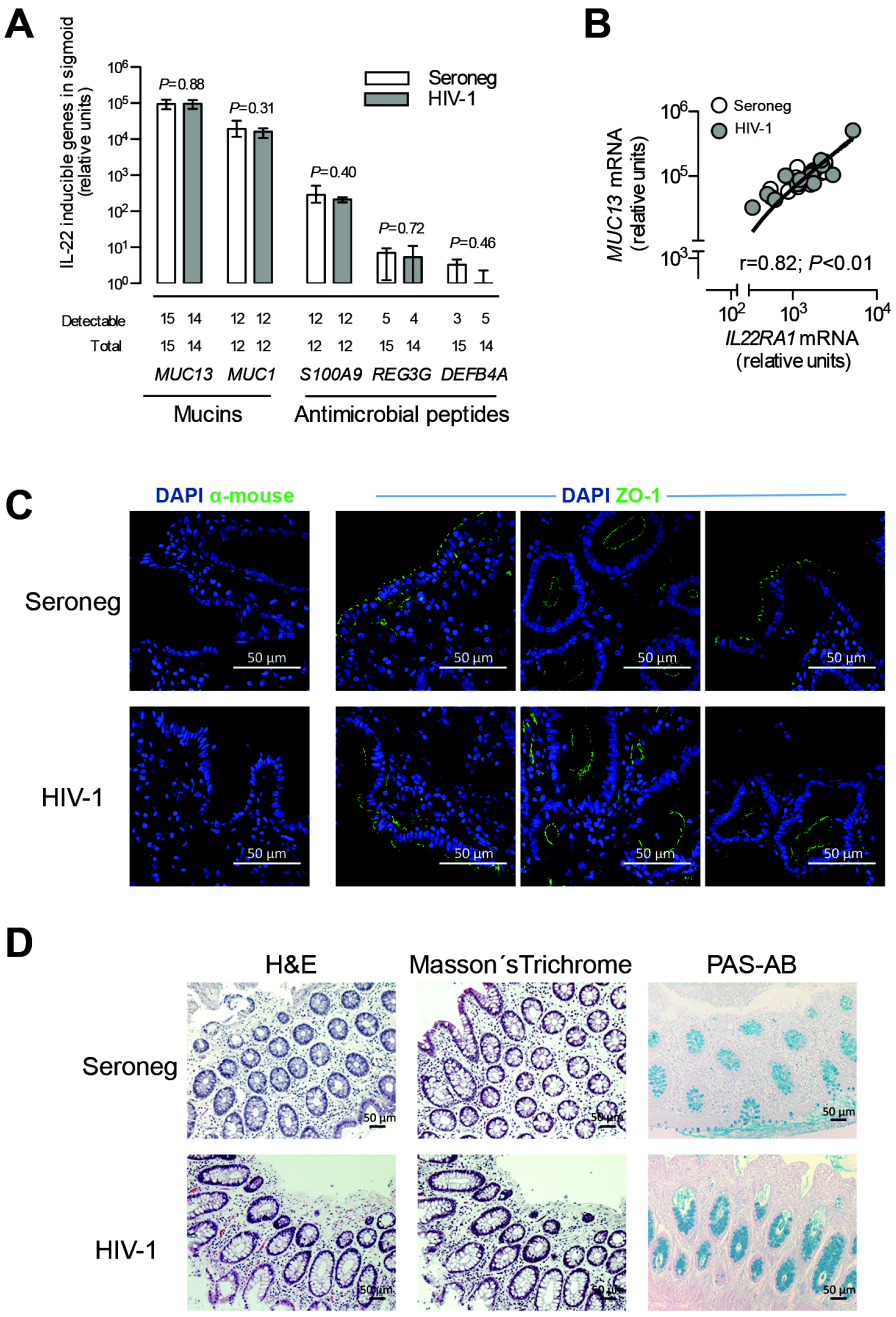

Figure 6. ***Levels of expression of IL-22-induced genes and sigmoid mucosa integrity.*** A) mRNA levels of mucins and antimicrobial molecules induced by IL-22 in biopsies from HIV-1 and control cohorts. Numbers indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*, with bars representing medians and interquartile range. Comparisons were made using Mann-Whitney U test; and *P* values are shown. B) Association between levels of expression of *IL22RA1* and *MUC13* mRNA. Spearman correlation was used to calculate r coefficient and *P* values. C) Illustrative immunofluorescence staining of ZO-1 in three different HIV-1-infected and seronegative individuals, used to quantify the proportion of epithelial area staining positive for ZO-1 in HIV-1 (21% [16-30], n=7) and control (22% [20-28], n=5) groups. D) Representative Haematoxylin and eosin (H&E), Masson's trichrome, and PAS-AB staining of sigmoid biopsies from HIV-1-infected and control individuals, used to score the histological alterations: mean value of 1.1 for HIV-1+ (n=13) and 1.4 for seronegatives (n=15). Bars = 50µm.

The strong association we observed between the levels of transcripts for *IL22RA1* and *MUC13* (Figure 6B) is worth emphasizing, as it supports the role of IL-22 in the induction of this particular molecule. Ileum biopsies from both cohorts also featured similar levels of these transcripts (see Supplementary Figure 2). Furthermore, there was no evidence of compromised tight junction integrity, as assessed by the levels of expression of *Zonula Occludens* (ZO)-1 (Figure 6C).

Additionally, blinded examination of H&E, Masson's trichrome and PAS-AB staining revealed no significant differences between the two cohorts with respect to crypt morphology, number and type of cell infiltrates and number of epithelial mitotic events (Figure 6D). Mucin production was apparently preserved in all samples, and significant collagen deposition was only found in biopsies from two HIV-1 individuals (data not shown).

Interestingly, our long-term treated HIV-1 cohort showed no significant increase in the expression of activation markers in circulating CD4 and CD8 T-cell subsets, or an expansion of $CD16^{+}$ cells within $CD14^{bright}$ monocytes (Table 1). The lack of up-regulation of these markers, that have been associated with microbial translocation, was in agreement with preserved epithelial integrity, as suggested by our histological data and the levels of antimicrobial peptides.

Altogether, our study of long-term treated HIV-1 infection provides evidence for full epithelium integrity in the context of significant Th22 depletion and preserved ILC3.

Discussion

Maintenance of intestinal homeostasis is linked to IL-22 production in the lamina propria[26]. This study provided evidence favouring distinct regulation of the two main IL-22 producers in the human gut, CD4 T-cells (Th22) and innate lymphoid cells (ILC3). We showed that IL-22-mediated processes were functioning in long-term treated HIV-1-infected patients, despite evidence of Th22 depletion. Conversely, innate IL-22-production was unaltered, suggesting a role for ILC3 in ensuring epithelial integrity.

Gut disruption is a hallmark of HIV-1 infection, and the restoration of intestinal integrity upon ART is still controversial[18]. Using a comprehensive approach combining tissue structure analyses, cell counts and relative proportion of functionally characterized populations, as well as quantification of key genes implicated in gut homeostasis, we demonstrated the absence of major GALT disturbances in patients starting ART even in advanced stages of the immunodeficiency. In summary, we documented: 1) an absence of CD4 T-cell depletion, reaching normal absolute counts in the lamina propria despite the persistence of increased CD8 T-cell numbers; 2) normal numbers, as well as balance, of regulatory T-cells and CD4 T-cells that produce the pro-inflammatory cytokines IL-17 and/or IFN-γ; 3) evidence of control of local inflammation provided by lack of over-expression of relevant genes (e.g. IL6, IL1B, TGFB1, and IDO1); 4) preserved epithelial structure in the sigmoid and ileum; and 5) absence of systemic markers associated with microbial translocation, in our long-term treated HIV cohort.

Importantly, our study revealed the maintenance of several processes that are known to rely on IL-22, namely ZO-1-associated tight junction integrity and production of regulins, S100, defensins and mucins, all of which are essential for preserving intestinal homeostasis[38]. This central role of IL-22 in tissue repair is in line with several reports in other clinical contexts and models of disease[26].

Strikingly, we documented mucosal integrity in spite of the significant Th22 depletion in the sigmoid mucosa of long-term treated HIV-1-infected individuals. To our knowledge, there is only one report evaluating IL-22-production in the human

sigmoid mucosa[27]. This cross-sectional study described a Th22 depletion associated with disruption of epithelial integrity that was apparently recovered in treated patients. However, this recovery should be cautiously interpreted as only 8, much younger controls were evaluated. This is particularly critical given our finding of a positive correlation between the frequency of Th22 within CD4 T-cells and age in seronegatives. Thus, the use of a large cohort of age-matched controls strengthens our observation of Th22 depletion after long-term ART in HIV-1 infection.

This preferential Th22 depletion could not be linked to an altered expression of factors that distinctly govern IL-22 and IL-17 production, namely AHR[24] and TGFβ[25]. The possibility of distinct homing requirements should be explored in future studies.

Interestingly, irrespective of the HIV infection status, our parallel study of circulating and mucosal Th22 cells revealed higher co-production of IL-17 and/or IFN-γ in the latter. It is plausible that cytokine patterns are influenced by the mucosal environment, in which case the similarity of the Th22 profile in the two cohorts represents an additional argument in favour of gut integrity in long-term treated HIV-1 patients. In agreement, no skewing towards IFN-γ production was found in ILC3 of the HIV-1 cohort, in contrast with published data on untreated SIV infection in non-human primates[39].

Importantly, ILC3 were not depleted in the treated HIV-1 cohort, supporting a role for distinct factors governing the homeostasis of the two main IL-22-producing subsets in the gut mucosa. Given the lower frequency of these cells, and the limited amount of tissue obtained during human studies, data on the relative contribution of ILC3 and Th22 to IL-22 production is still scarce. In contrast, murine models of colitis have generated data in favour of distinct regulation of these two subsets during disease[40]. Moreover, ILC were shown to be able to maintain epithelial preservation in the absence of IL-22 production by T-cells[40]. Thus, it is plausible that the relative proximity to epithelial cells, rather than the actual frequency in lamina propria, may determine ILC's part in maintaining epithelial integrity and also help preserve epithelial stem cells[3]. Nevertheless, although we were able to identify ILC3 in human

gut, biopsy structure in frozen tissue was insufficiently well preserved to allow us to assess their relative localization in relation to Th22. Notably, in addition to IL-22 production, ILC3 were recently reported to control mucosal inflammation by modulating local CD4 T-cell activation[15]. Thus, ILC3 may represent an important population to target in the context of HIV-1 infection, since a hyper-activated state is believed to underlie mucosal damage.

Overall, mucosal homeostasis, including IL-22-mediated mechanisms essential for epithelial integrity, could mainly rely on innate lymphoid cells, as suggested by our data on long-term treated HIV-1-infected patients. Thus, the therapeutic targeting of ILC may represent an important strategy to promote intestinal recovery.

Author contribution: Performed research: S.M.F., A.R.P., C.F., J.R. and RB; Clinical data and sample collection: S.M.F., L.C., C.S. J.P.; Designed the study, analysed data and wrote the paper: S.M.F., R.B., H.V.F. and A.E.S.

Conflict of Interests: The authors have no conflict of interest to report.

Funding: This work was supported by the Fundação para a Ciência e Tecnologia (FCT), and the Programa Operacional Ciência e Inovação 2010 [PIC/IC/82712/2007 to A.E.S.]. S.M.F., A.R.P and R.B.F received FCT scholarships.

Acknowledgements

We would like to thank the individuals that participated in the study, as well as the physicians for their follow-up and collection of clinical data, particularly Joana Fernandes, Manuela Doroana, Sérgio E. Paulo, Nuno Janeiro, and Francisco Antunes from the Department of Infectious Diseases of Hospital de Santa Maria, Lisbon; the collaboration of the Gastroenterology Departments of Hospital de Santa Maria, Lisbon, and Hospital de S. Bernardo, Setúbal, for the endoscopic studies; Paula Matoso and Vânia Silva for the technical assistance in molecular biology; and Bruno Silva-Santos, Helena Nunes-Cabaço, Iris Caramalho, and Rui M.M. Victorino for the critical discussion of the manuscript.

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Supplementary data

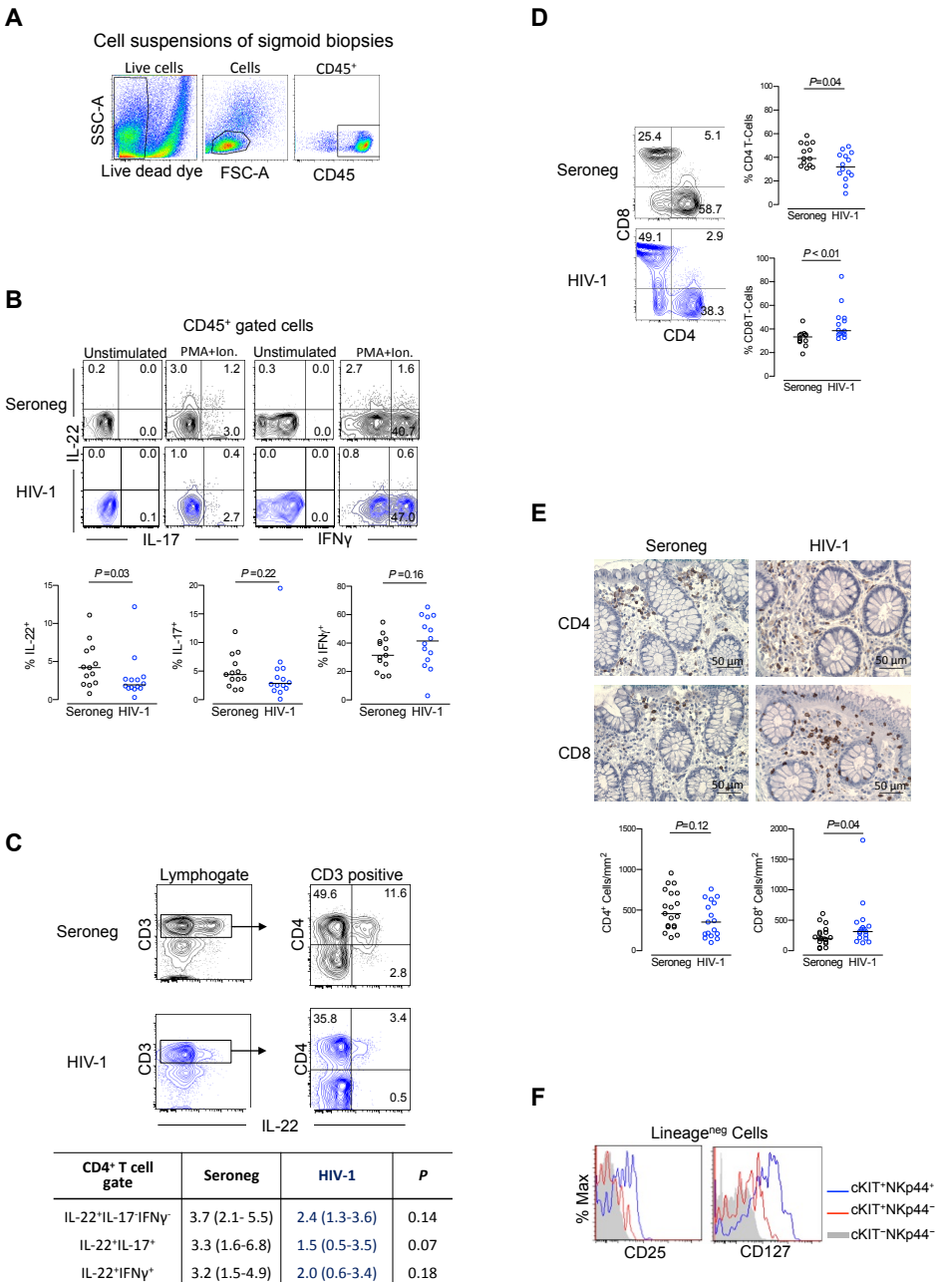
Supplementary Table 1. Antibodies used

Monoclonal Antibodies used in Flow Cytometry Staining			
Antibody	Fluorochrome	Clone	Source
IL-17	FITC	eBIO54DEC17	eBioscience[a]
IL-22	PE	22URTI	eBioscience
FOXP3	V450	PCH101	eBioscience
CD117 (cKIT)	PerCP	104D2	eBioscience
CD45	V450	2D1	eBioscience
CD45RO	APC-Cy7	UCHL1	eBioscience
CD3	V500	UCHT1	eBioscience
CD4	APC	RPA-T4	eBioscience
CD8	PerCP	RPA-T8	eBioscience
CD14	PE-Cy7	61D3	eBioscience
CD19	APC-efluor780	HIB19	eBioscience
CD16	FITC	eBioCB16	eBioscience
CD38	PE	HB7	eBioscience
HLA-DR	PerCP-efluor 710	L243	eBioscience
IFNy	PE-Cy7	4S.B3	Biolegend[b]
CD123	V450	6H6	Biolegend
Nkp44	APC	243415	R&D[c]
CCR7	FITC	150503	R&D
CD25	PE-Cy7	M-A251	BD Bioscience[d]
CD45RO	APC-H7	UCHL1	BD Bioscience
CD56	PE	My31	BD Bioscience
CD11c	APC	B-Ly6	BD Bioscience
CD3	V450	UCHT1	BD Bioscience
CD4	V500	RPA-T4	BD Bioscience

Antibodies used in Immunofluorescence Staining		
	Antibody	Source
Primary antibodies	Rabbit anti-RORγt	Abcam[e]
	Goat anti-IL-22	Santa Cruz Biotechnology[f]
	Rat anti-CD3	Acris[g]
	Mouse anti-ZO-1	BD Biosciences
	Alexa Fluor 488 Anti-Goat	Molecular Probes[h]
Secondary antibodies	Alexa Fluor 488 Anti-Mouse	Molecular Probes
	Alexa Fluor 546 Anti-Rabbit	Molecular Probes
	Alexa Fluor 647 Anti-Rat	Molecular Probes

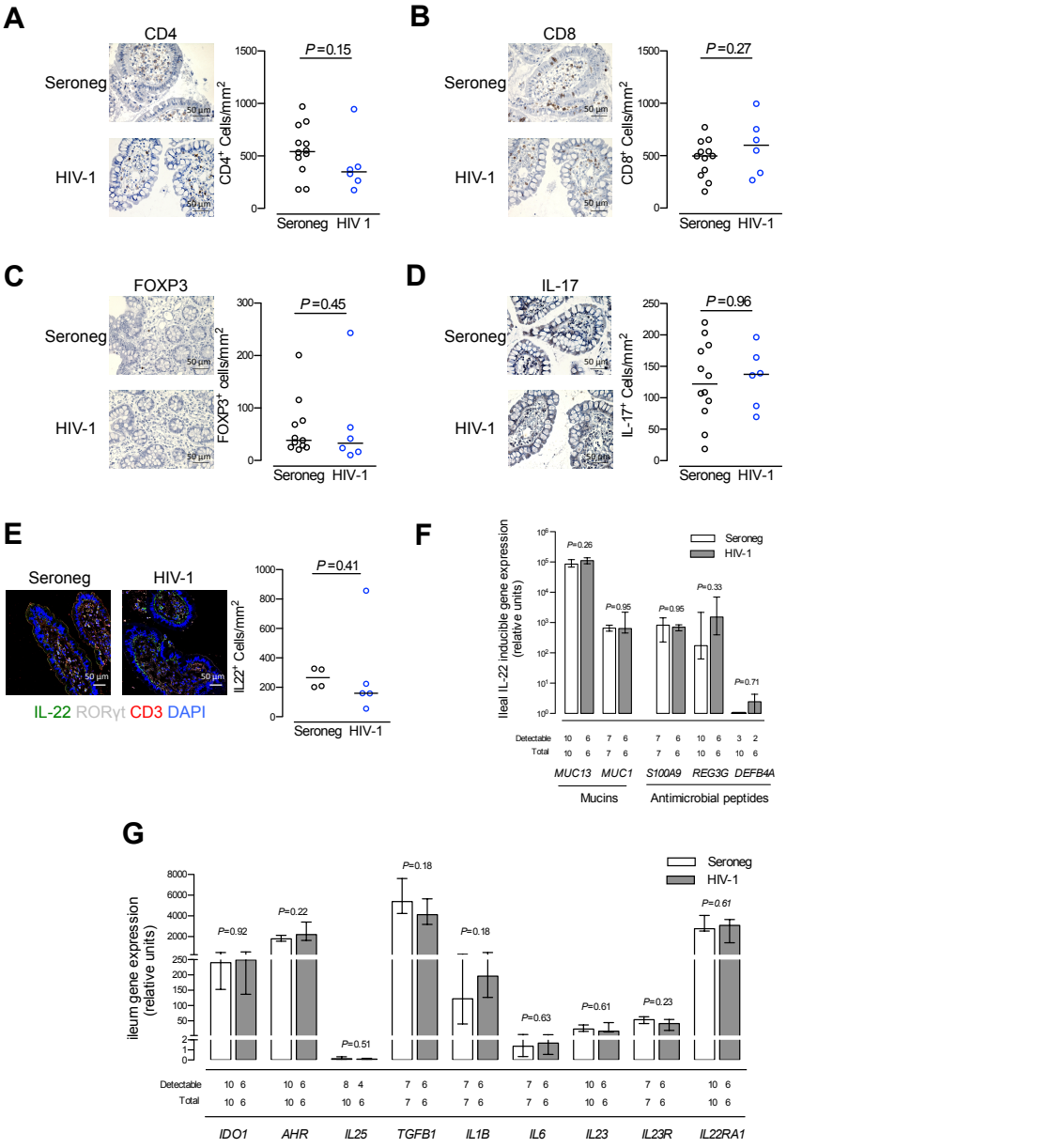
Antibodies used in Immunochemistry Staining		
Antibody	Clone	Source
CD4	4B12	Leica Biosystems[i]
CD8	1A5	Leica Biosystems
FOXP3	236/E7	Leica Biosystems
IL17	polyclonal	Santa Cruz Biotechnology

[a]eBioscience, San Diego, CA; [b]Biolegend, San Diego, CA; [c]R&D, Minnneapolis, MN, USA; [d]BD Bioscience, San Jose, CA; [e]Abcam, Cambridge, UK; [f]Santa Cruz Biotechnology, Dallas, USA; [g]Acris, Herford, Germany; [h]Molecular Probes, Life Technologies, Carlsbad, USA; [i]Leica Biosystems, Wetzlar, Germany.



Supplementary Figure 1. ***Analysis of sigmoid mucosa****. A)* Flow cytometry analysis of mucosal cells illustrating the sequential gating strategy of cell suspensions obtained from sigmoid biopsies of macroscopically normal mucosa showing dot-plots of dead cell exclusion, lymphogate definition accordingly to SSC-A and FSC-A, and gating on CD45+ cells. B) Representative dot-plots of IL-22, IL-17 and IFN-γ staining within live CD45+ population, with and without PMA-ionomycin stimulation, in a seronegative and an HIV-1-infected individual, with graphs showing the frequency of cytokine producing cells in HIV-1-infected (n=14) and seronegative (n=13) cohorts. C) Illustrative dot-plots of IL-22 and CD3 staining within lymphogate, as well as of IL-22 and CD4 staining within total T-cells in a seronegative and an HIV-1-infected subject; the frequency of the IL-22-producing subpopulations within total CD4 T-cells in HIV-1-infected (n=13) and seronegative (n=13) cohort is tabulated bellow. D) Representative dot-plots of CD4 and CD8 expression analysed within total CD3+ cells in a

seronegative and an HIV-1-infected subject; graphs show the frequency of these subsets in seronegative (n=13) and HIV-1-infected (n=14) cohorts.
E) Representative immunohistochemistry images of CD4 and CD8 staining (black bars indicate 50μm) in a seronegative and an HIV-1-infected subject; graphs show the corresponding counts of CD4 and CD8 positive cells in seronegative (n=19) and HIV-1 (n=17) cohorts. F) Expression of the α-chains of the IL-7 receptor (CD127) and the IL-2 receptor (CD25) by innate lymphoid cells isolated from the sigmoid mucosa of an HIV-1-infected individual; analysis was performed after gating in lineage (CD3, CD19, CD14, CD16, CD123) negative cells, and histograms compare the expression of CD127 and CD25 within $cKIT^{+}NKp44^{+}$, $cKIT^{+}NKp44^{-}$ and $cKIT^{-}NKp44^{-}$ cells. In the dot-plots, numbers inside quadrants represent the frequency of positive cells. In the graphs, each dot represents an individual, and bars indicate medians. Analysis was performed using Mann-Whitney U test and all *P* values are shown.



Supplementary Figure 2. ***Analysis of ileum mucosa.*** Ileum biopsies obtained from 6 HIV-1-infected and 12 seronegative individuals, comparable in terms of age (54 years [52-60] versus 55 years [49-60], respectively) and CD4 counts (580 cells/µL [411-630] versus 672 cells/µL [584-822], respectively), with an over-representation of males in the HIV-1 group (5/6 versus 5/12). Representative images of CD4 (A), CD8 (B), FOXP3 (C) and IL-17 (D), and related cell counts in the ileum mucosa of HIV-1-infected (n=6) and control individuals (n=12) are shown. E) Illustrative immunofluorescence staining of IL-22, RORγt and CD3 in the ileum mucosa of HIV-1 and seronegative subjects. Total number of IL-22$^+$ cells/mm^2 per group are shown. Ileum mRNA levels of genes induced by IL-22 (F), and of genes known to modulate IL-22 production and/or function (G), with numbers of samples evaluated and of samples with detectable levels of the respective genes depicted below. Results are expressed in relative units, normalized to the mean CT levels of GAPDH and r18S. Each dot represents an individual and bars represent medians in the graphs. Bar graphs represent medians and interquartile range. Comparisons were made using Mann-Whitney U test and *P* values are shown.

3.2. The gut in HIV-2 infection

3.2.1. Gut disruption in HIV-2 infection despite reduced viremia

Gut disruption in HIV-2 infection despite reduced viremia

Susana M. Fernandes[1,2], Ana R. Pires[1], Cristina Ferreira[3], Rita Tendeiro[1], Luís Correia[4], Sérgio E. Paulo[5], Rui M.M. Victorino[1,2], Ana E. Sousa[1]

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Running title: HIV-2-associated Mucosal CD4 Depletion

Key Words: HIV-2; Immune Reconstitution; Gut-associated Lymphoid Tissue; Immune Activation; Antiretroviral Therapy.

Published:
Susana M. Fernandes, Ana R. Pires, Cristina Ferreira, Rita Tendeiro, Luís Correia, Sérgio E. Paulo, Rui M.M. Victorino, Ana E. Sousa.
Gut disruption in HIV-2 infection despite reduced viremia.
AIDS. Jan 2014; 28:290-2.

HIV-2 infection is highly prevalent in West Africa, and has been increasingly observed in non-African countries, mostly associated with migratory populations[1]. It has a much more benign course and lower viremia than HIV-1[2], though with similar clinical spectra. Half of the HIV-2-infected patients with less than 200 CD4 T-cells/µl exhibit undetectable viremia, despite harbouring numbers of infected cells comparable to their HIV-1 counterparts[3]. Moreover, CD4 T-cell loss occurs in direct association with progressive immune activation in both infections, though the depletion rate is much slower in HIV-2[2, 4].

HIV-1 disease progression has been linked to disruption of gut-associated lymphoid tissue (GALT) and increased levels of microbial translocation, leading to systemic immune activation. There are currently no data on the impact of HIV-2 on GALT.

Here, we provide evidence of HIV-2 replication in the gut despite the low viremia, which was associated with major mucosal disruption and CD4 T-cell depletion that recovered upon antiretroviral treatment (ART).

An 18-yr-old male, from Guinea-Bissau, with HIV-2 infection possibly acquired by vertical transmission, presented with mild diarrhea since the first year of life. Colonoscopy showed loss of haustra and rectal ulcers, with moderate lymphoplasmocytic and neutrophilic infiltrates in rectal biopsies (Figure 1A-B). Circulating CD4 T-cells were low (19%, 103 cells/µL), in association with hyper-immune activation (Figure 1C) and detectable viremia (4,575 RNA copies/mL), albeit at levels much lower than those found in HIV-1 infection. There was a significant depletion of CD4 T-cells and an increase in CD8 T-cells in sigmoid lamina propria (Figure 1A). This ratio inversion was associated with increased regulatory T-cells (Treg, FOXP3+) and decreased interleukin (IL)-17-producing cells (Figure 1A), an immunological profile strikingly similar to that described for HIV-1-infected individuals.

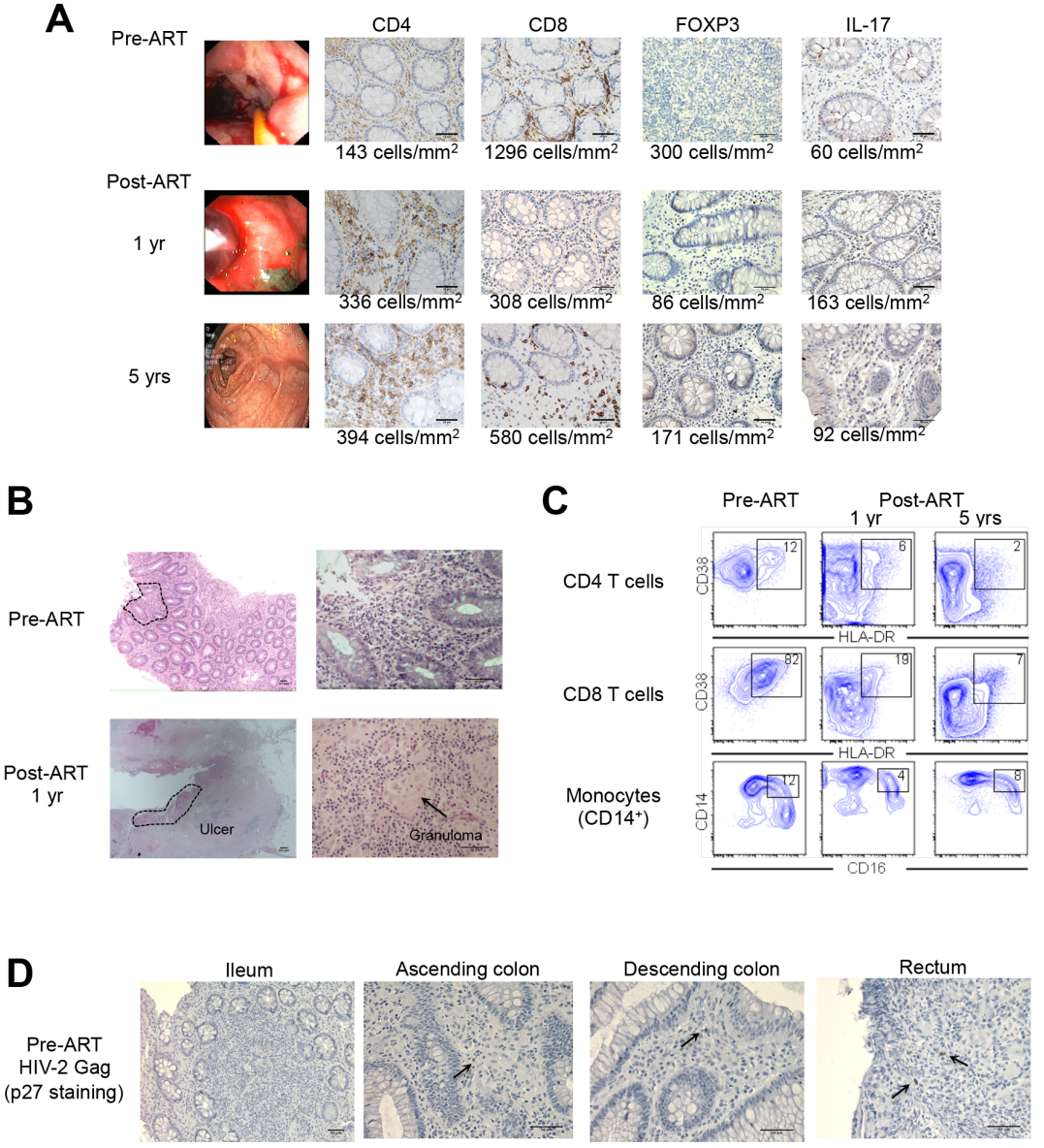

Figure 1. ***Gut disturbances in an HIV-2 infected patient, and their recovery upon ART.*** A. Longitudinal evaluation of sigmoid mucosa: colonoscopy images and lamina propria lymphocyte populations assessed by immunohistochemistry (reference healthy controls' counts/mm²: CD4+: 501±56, n=19; CD8+: 202±36, n=19; FOXP3+: 69±19, n=17; IL-17+: 123±26, n=15). B. Haematoxylin-eosin staining of colon biopsies collected pre-ART and of surgical piece (1yr post-ART). Dashed lines highlight inflammatory infiltrates and ulcerated regions. C. Immune activation markers assessed by flow cytometry, numbers inside gates indicate frequency (reference healthy controls' frequencies, n=16: 1.2±0.1 and 4.4±1.4 of HLA-DR+CD38+ within CD4 and CD8 T-cells, respectively; 4.6±0.7 of CD16+CD14bright cells within monocytes). D. Levels of viral replication in the gut pre-ART assessed by immunohistochemistry against HIV-2 Gag (anti-SIV p27 which cross-reacts with HIV-2 p26, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from Dr. P. Szawlowski). Arrows indicate positive cells. (Bars = 50 µm).

These gut disturbances were accompanied by an increase in serum markers usually linked with high levels of microbial translocation, assessed as previously described[5]: plasma lipopolysaccharide (LPS, 136 pg/mL), serum LPS binding-protein (LBP, 1.2µg/mL) and soluble-CD14 (3.2 µg/mL), as well as systemic immune activation (Figure 1C). Thus, our findings support a contribution of microbial translocation to HIV-2 pathogenesis, adding to this ongoing debate[5, 6].

Local viral replication is considered a main determinant of HIV-1-associated mucosal disturbances. We found expression of HIV-2 Gag protein in different colon segments (Figure 1D), which supports a significant degree of mucosal HIV-2 replication, despite the low viremia. This is in line with our previous findings of ongoing viral replication in HIV-2 patients with undetectable circulating virus, and similar levels of cell-associated viral burden in the two infections[3].

ART (emtricitabine/tenofovir/saquinavir/ritonavir) was initiated with clear virological (undetectable viremia) and immunological responses (845 CD4 T-cells/µL, 25.5%; 1-year post-ART), accompanied by progressive decline in systemic activation markers (Figure 1C). Notably, our patient presented a marked delay of puberty (bone age of 12.5years), and, like HIV-1 infected children, an interstitial lymphoid pneumonia that fully resolved with therapy.

Conversely, diarrhea persisted with exacerbation of inflammatory histologic findings (transmural chronic inflammatory infiltrate with lymphoid aggregates, sparse noncaseating granulomas and mucosal architectural distortion), mimicking Crohn's disease, leading to the development of rectal stenosis that required endoscopic dilatations and segmental resection (Figure 1A-B). In spite of these disturbances, there was a recovery of lamina propria lymphocyte imbalances (Figure 1A), as well as lack of evidence of HIV-2 replication (Gag expression, data not shown). It is plausible that this paradoxical evolution represents a form of late immunological reconstitution disease, as at that time there was already a recovery of mucosal CD4 T-cells and IL-17 production (Figure 1A). Gut manifestations disappeared after prolonged ART, with sustained immunological response, both in gut mucosa (Figure 1A) and peripheral blood (716 CD4 T-cells/µL, 48.1%; 5-years post-ART).

Thus, our results represent a rational basis for starting ART in HIV-2 infection irrespectively of viremia. There are currently no randomized trials addressing ART in HIV-2 infected individuals[1]. Longitudinal studies suggest that HIV-2 infected patients have a limited response to ART, specifically a reduced CD4 T-cell recovery as compared to HIV-1[2, 3]. This might be related to virologic inefficacy, since antiretroviral drugs have not been specifically designed for HIV-2, and genotypic and phenotypic resistance studies are limited precluding an adequate choice of the best regimens[1]. Alternatively, the prolonged course of HIV-2 disease might lead to irreversible damage of secondary lymphoid organs, thereby preventing complete immunological recovery.

In conclusion, this is the first report of mucosal CD4 T-cell depletion with loss of IL-17-producing cells in HIV-2 infection, which was shown to recover upon suppression of ongoing low-level viral replication with ART. Our results represent an argument in favor of treating HIV infection in the context of reduced viremia.

Author contributions: Performed research: S.M.F., A.R.P., C.F., and RT; Clinical data collection: S.M.F., L.C., and S.E.P.; Designed the study, analysed data and wrote the paper: S.M.F., R.M.M.V., and A.E.S.

Conflict of interests: The authors have no conflict of interest to declare.

Funding: This work was supported by the Fundação para a Ciência e Tecnologia (FCT), and the Programa Operacional Ciência e Inovação 2010 (PIC/IC/82712/2007 to A.E.S.). S.M.F., A.R.P, and R.T. received FCT scholarships.

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3.2.2. Mucosa CD4 T-cells and gut integrity are preserved in chronic HIV-2 infection

Mucosa CD4 T-cells and gut integrity are preserved in chronic HIV-2 infection

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Manuscript in preparation

Abstract

HIV-2, the second AIDS virus, causes an attenuated form of disease characterized by low to undetectable viremia and slow CD4 decline in association with progressive hyper-immune activation. We hypothesized that the slower evolution of immune activation and consequent protracted course of HIV-2 disease may be related to preservation of the gut mucosa.

We performed, for the first time, a comparative analysis of colon biopsies from HIV-2-infected individuals (n=13) with depletion of circulating CD4 T-cells accompanied by over-expression of activation markers despite undetectable viremia, and seronegative controls (n=20), submitted to colon cancer screening colonoscopies. The sigmoid mucosa from HIV-2-infected individuals featured an expansion of IFN-γ-producing CD8 T-cells but no depletion of CD4 T-cells. We also observed no changes in the relative proportion or counts of Foxp3+, as well as IL-17- and IL-22-producing CD4 T-cells. Of note samples from HIV-2-infected individuals showed significantly increased transcriptional levels of chemokines linked to gut CD4 trafficking. Additionally, the IL-22-producing innate lymphoid cell compartment was maintained. In agreement, we found evidence that epithelial integrity was unperturbed; namely a lack of structural alterations or collagen deposition and preserved levels of the tight junction protein *Zonula Occludens* (ZO)-1 and transcriptional levels of antimicrobial peptides and mucins. Furthermore, gut integrity remained unaltered in individuals with evidence of local HIV-2 replication (Gag immunohistochemistry).

Overall, we demonstrated that chronic HIV-2 infection is associated with lack of CD4 T-cell depletion in the gut mucosa and preserved epithelial integrity. These findings may explain the relatively benign course of HIV-2 disease. Moreover, we found that the mucosal CD4 T-cell homeostasis is likely to be ensured by a local response, with over-expression of critical homeostatic chemokines, which have implications for the design of therapies targeting the recovery of the gut in HIV-1 infection.

Author Summary

The loss of CD4 T-cells at the mucosal level and the associated disruption of gut integrity are currently considered main determinants of the inflammatory state that characterizes HIV/AIDS and a main cause of morbidity in treated HIV-1 infected individuals. HIV-2 infection is associated with a much slower rate of increase in immune activation levels and consequently a protracted CD4 T-cell decline, leading to a limited impact on the survival of untreated infected adults.

This first study of the gut mucosa of HIV-2-infected individuals revealed preserved CD4 T-cells, IL-17 and IL-22 production, and epithelial integrity, even in those with local HIV-2 replication. Mucosal CD4 T-cell homeostasis was partly ensured by the over-expression in the gut of chemokines implicated in mucosal CD4 T-cell recruitment. These data provide a possible explanation for the relatively benign course of HIV-2 infection, and have implications for the design of therapies targeting the recovery of the gut in HIV-1 infection.

Introduction

Unlike HIV-1, HIV-2 infection is associated with a more benign disease course, characterized by a reduced rate of CD4 T-cell depletion[1] and low to undetectable levels of viremia[2]. Nevertheless, CD4 T-cell loss does occur in HIV-2 infected individuals and has been linked to persistently increased levels of immune activation[2-4]. The factors driving this state of hyper-immune activation in the presence of reduced plasma viral load remain largely unclear, and it has been suggested to result from ongoing viral replication[5] and/or anti-viral responses[6].

One key area that has not been explored is the contribution of gut disruption to the generalized immune activation observed in HIV-2 infection. This is particularly relevant given that a hallmark of HIV-1 infection is marked gut mucosal disruption, a process linked to microbial translocation which in turn is thought to drive hyper-immune activation and progression to AIDS[7, 8]. HIV-1 infection results in a massive early depletion of gut mucosal CD4 T-cells[9] that persists throughout infection. Moreover, IL-17[10] and IL-22[11] producing cells are preferentially depleted, whilst mucosal regulatory T-cells (Tregs) increase[12]. The process of gut disruption may be aggravated by the ability of HIV-1 to directly deregulate epithelial integrity via impairment of epithelial tight junctions[13]. On the other hand, HIV-1 long-term non-progressors have strong mucosal HIV-specific responses and preserve mucosa CD4 T-cells and subsets[14-16].

A similar picture has emerged from primate studies. Pathogenic SIV infection is characterized by gut disruption in early infection and persistent CD4 T-cell loss, together with Th17/Treg imbalances[17, 18]. Additionally, IL-17 production by class 3 innate lymphoid cells (ILC3), a recently identified key subset for both the maintenance of gut epithelial homeostasis and the control of CD4 T-cell responses[19], was shown to be decreased in this context, due to a local over expression of indoleamine 2,3-dioxygenase[20]. Moreover, non-pathogenic SIV infection of its natural hosts, such as Sooty Mangabeys, does not seem to result in a perturbation of gut mucosal homeostasis, which in turn is associated with a lack of microbial translocation and immune activation[10].

Microbial translocation has been estimated in HIV-2 infected individuals by the measurement of lipopolysaccharide (LPS) in African and European cohorts, and generated conflicting results. Our group was unable to show a link between LPS levels and immune activation or CD4 T-cell loss in chronic HIV-2 infection[21]. Nevertheless, other data suggests that HIV-2-infected individuals also feature increased levels of microbial translocation, but only on reaching the AIDS stage of disease[22].

The restoration/normalization of gut mucosa homeostasis upon antiretroviral therapy (ART) remains the subject of debate[23]. We have shown that Th22 cells were not recovered on long-term treated HIV-1-infected individuals[24], where as others have shown persistent disruption of gut homeostasis[25, 26]. What is clear is that increased levels of immune activation persist even in fully responsive long-term ART-treated HIV-1-infected individuals[26], which may be linked to the failure to completely ablate viral replication[27, 28] and/or fully correct disturbances in gut mucosa homeostasis[25, 26]. Irrespective of the cause, persistent immune activation has been linked to the increased morbidity observed in these individuals. Thus, HIV/AIDS continues to represent a major health problem even in countries with access to ART, highlighting the importance in obtaining a better understanding of the local response to HIV infection in the gut, which can help to develop novel interventions to help fully restore gut mucosa homeostasis.

We investigated here the impact of HIV-2 infection on gut mucosal homeostasis through a comprehensive analysis of colon biopsies from a cohort of ART-treated and untreated HIV-2-infected individuals. We found no apparent depletion of sigmoid CD4 T-cell and a maintenace of Th17 and Th22 compartments in the absence of increased regulatory T-cells in the sigmoid mucosa of HIV-2-infected as compared to seronegatives. Moreover, the ILC3 compartment was preserved, as was innate cell production of IL-22 and IL-17. Finally, mucosal structure and epithelial integrity appeared normal with no reduction in the expression of relevant epithelial genes and maintained expression of the tight junction protein ZO-1. Thus we provide evidence of persevered gut mucosal homeostasis in HIV-2 infection, possibly explaining the slow rate of increase in immune activation associated with this natural model of attenuated HIV disease.

Methods

Studied cohorts

The study enrolled 13 HIV-2-infected individuals and 20 seronegatives (control cohort previously described[24]) with clinical indication to perform colonoscopy (31/33 colon cancer screening) that were found to be cancer free with the exception of one HIV-2 patient that had a rectal adenocarcinoma (Table 1). All individuals gave written informed consent for blood sample and gut biopsy collection and processing. Study was conducted under approval of the Ethical Boards of the Faculty of Medicine of University of Lisbon, Hospitals Santa Maria and São Bernardo.

Cell isolation

Twelve sigmoid biopsies per individual were collected from macroscopically normal mucosa, and processed immediately. 7-10 biopsies were digested with collagenase B (10mg/mL, Roche, Penzberg, Germany), at 37ºC, and then mechanically macerated. Lymphocytes were separated by Percoll gradient, and rested for 16 hours at 37ºC in complete medium. Blood was collected in parallel from 10 HIV-1-infected and 9 control individuals and peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Hypaque centrifugation gradient.

Flow cytometry

Cells were surface and intracellularly stained (Foxp3 Staining Buffer Set, Ebioscience), as previously described[29], using a panel of antibodies (S1 Table), and acquired on LSRFortessa flow cytometer (BD Biosciences). Circulating lymphocyte populations were assessed using whole blood staining, and analysed for activation markers as previously described[4, 21]. Cytokine production was assessed after 4-hour stimulation with phorbol myristate acetate (PMA), plus ionomycin,[30], using unstimulated cells to define cytokine cut-offs. The following antibody combinations were used to evaluate: 1) mucosal ILC - IL-17/IL-22/cKIT/IFN-γ/NKp44/LD/LIN/CD45; 2) mucosal T-cell subsets - IL-17/IL-22/CD8/IFN-γ/CD4/LD/FOXP3/CD3; 3). cytokine production by PBMC - CCR7/IL-22/IL-17/IFN-γ/CD8/CD45RO/IL-2/CD3. Analysis was performed with FlowJo (version 9.3.1, Tree

Star, Inc, Ashland, OR), after exclusion of dead cells (Live/Dead-LD, Life Technologies, Carlsbad, CA), within gated populations containing ≥150 events.

Immunohistochemistry staining

One sigmoid biopsy was embedded in paraffin after preservation in 4% formaldehyde. 3µm sections were stained with antibodies (S2 Table), after paraffin removal with xylene, rehydration with alcohol, and antigen retrieval by heat. As a positive control for HIV-2 Gag staining, a Tonsil Organ culture (TOC) was infected in vitro with HIV-2 ROD for 7 days, washed and subsequently processed as described above. Cells were counted manually in ten images (400x) acquired with a Leica DM2500 brightfield microscope, and lamina propria area assessed using ImageJ 1.47i (NIH, USA), in blinded samples by two researchers. Haematoxylin and eosin (H&E), Masson's Trichrome and Periodic Acid Shiff – Alcian blue (PAS-AB) stainings were concurrently performed, and blindly evaluated by a trained pathologist. Samples were scored according to inflammatory alterations, type of infiltrate, presence or absence of fibrosis, and number of mitotic figures at the crypt base with scores ranging from 0 to 3.

Immunofluorescence staining

One sigmoid biopsy was frozen in OCT (VWR, Radnor, PA) as previously described[24]. 3µm tissue sections were stained by sequential antibody incubations (S3 Table), after protein blocking. DAPI was used for nuclear counterstaining. Images were acquired with Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a Plan-Apochromat 20x/0.8 objective, surface area measured with ImageJ 1.47i, and positive cells counted using in-house software, written in MATLAB (Mathworks, Natick, MA), allowing automatic generation of individual report images for cell-counting verification[24].

RNA Extraction and analysis

One sigmoid biopsy was stored in RLT-buffer (Qiagen, Valencia, CA) immediately post-collection. RNA was extracted using Allprep RNA/DNA mini kit (Qiagen), and 250ng used to synthesize cDNA (SuperScript III, Life Technologies). Expression levels of *IL22*, *IL17A*, *IL22RA1*, *IL23*, *IL23R*, *IL25*, *TGFB1*, *IL1B*, *IL6*, *IL10*,

CCL20, CCL28, GMCSF, *MUC1*, *MUC13*, *S100A9*, *REG3G*, *DEFB4A*, *AHR* and *IDO1* were measured after pre-amplification with TaqMan Preamp Master Mix, using TaqMan gene expression assays with an Applied Biosystems 7500 Fast Real-Time PCR System (all from Life Technologies). Results are expressed as delta CT normalized to the medium CT levels of *GAPDH* and *r18S*. When gene expression was undetectable, a value 40 CT was used for the purposes of statistical analysis.

Plasma viral load

HIV-2 viremia was quantified by RT-PCR using a previously described assay[31], with a detection cut-off of 40 RNA copies/ml.

Statistical Analysis

Results are expressed as medians and interquartile range. Comparisons between two groups were made using Mann-Whitney U test, using Graph Prism version 5.0 (GraphPad Software, San Diego, CA). Non-adjusted regression analysis or Spearman correlation was used to identify associations between several parameters and multivariate linear regression analysis used to adjust results for race using STATA 12.1 (StataCorp, College Station, Texas, USA). Analysis and presentation of distributions of cytokine production by $CD4^+$ T-cells was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov. Comparison of cytokine-producing subset distributions was performed using Wilcoxon Signed Rank test and partial permutation test[32]. *P* values below 0.05 were considered significant.

Results

Preservation of mucosal CD4 T-cells in HIV-2 infected individuals

Sigmoid biopsies collected from 13 HIV-2 infected individuals during routine colon cancer screening colonoscopies were evaluated in parallel with those obtained from a cohort of 20 seronegatives, previously described in a study of long term-tretated HIV-1 infected individuals[24]. The HIV-2 cohort featured a statistically significant expansion of CD8 T-cells and increased levels of immune activation markers within CD4 and CD8 T-cells, as well as monocytes, despite of the lack of detectable viremia in all individuals, irrespective of ART (Table 1).

Table 1. Cohort Characterization

	Seronegative	HIV-2
Number [male/female]	20 [7/13]	13 [4/9]
Age	58 (49-63)	61 (58-65)
Race [white/black]	20/0	8/6
Length of follow-up (y)	NA	13 (7-19)
Patients on ART, n	NA	7
Length of ART[a] (y)	NA	3 (2-16)
Nadir CD4 T-cells/μL before ART	NA	307 (250-480)
Viremia (RNA copies/mL)	NA	< 40
CD4 T-cells/μL	619 (566-739)	490 (301-734)
CD8 T-cells/μL	375 (296-521)	654 (389-1057)[c]
Naïve ($CD45RO^{-}CCR7^{+}$)[b]		
% within CD4 T-cells	30.5 (25.8-41.5)	43.7 (33.9-49.3)
% within CD8 T-cells	16.8 (3.8-31.7)	10.9 (7.0-19.2)
$HLA\text{-}DR^{+}CD38^{+}$ [b]		
% within memory-effector CD4 T-cells	3.4 (2.8-4.6)	8.0 (6.2-14.8)[c]
% within memory-effector CD8 T-cells	11.0 (7.6-16.0)	44.8 (21.2-53.9)[c]
% 16^{+} within $CD14^{bright}$ monocytes [b]	7.5 (6.3-7.7)	11.0 (7.5-19.9) [c]
Memory-effector CD4 T-cells[b]		
% $IL\text{-}22^{+}$	1.5 (0.9-1.5)	0.9 (0.6-1.9)
% $IL\text{-}17^{+}$	0.7 (0.6-1.5)	1.2 (0.9-1.5)
% $IFN\gamma^{+}$	10.2 (6.8-38.8)	15.6 (11.2-20.3)

Data expressed as medians, interquartile range in brackets. Statistical analysis performed with Mann Whitney U test. [a]Triple therapy in all patients, always including protease inhibitors .[b]Peripheral blood analysis of 12 HIV-2-infected and 9 control individuals . [c]$P<.05$ to seronegative.

We found that both the frequency of CD4 T-cells within total lymphocytes measured by flow cytometry, and the number of CD4 T-cells counted in the lamina propria were not significantly different in HIV-2-infected and seronegative individuals (Fig. 1).

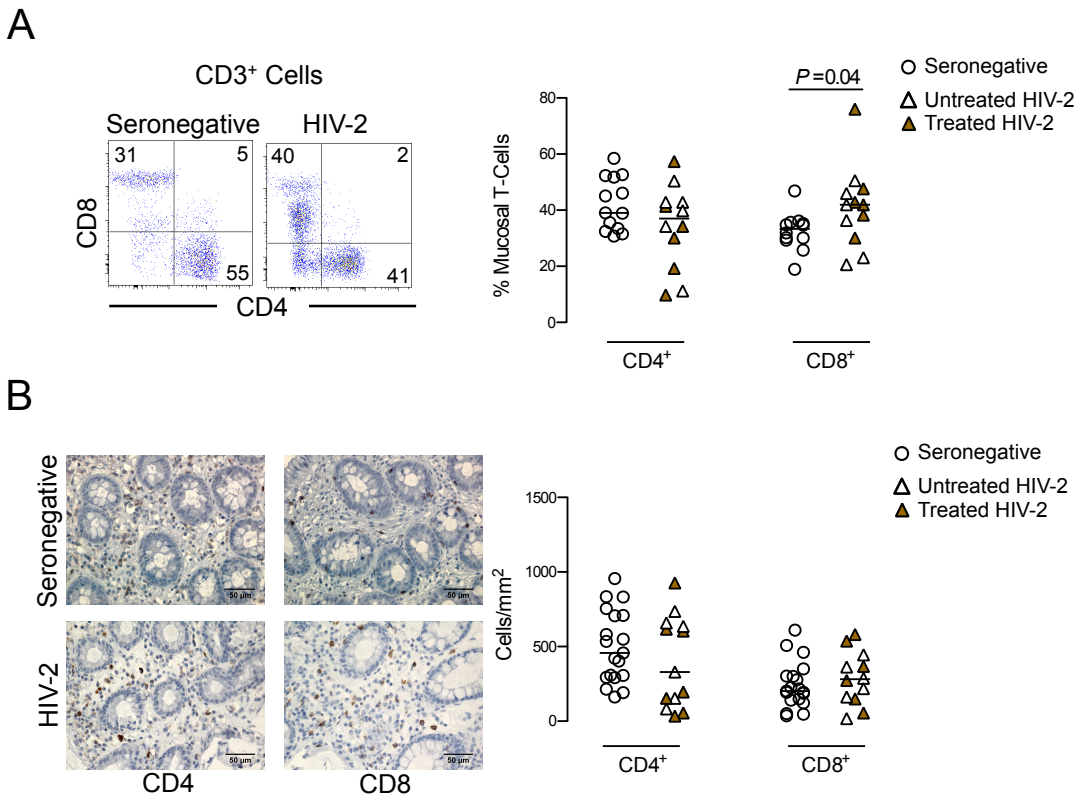

Figure 1. Gut mucosa T-cells. A. Flow cytometry analysis of cell suspensions generated from sigmoid biopsies of HIV-2-infected and seronegative individuals showing representative dot plots of CD4+ and CD8+ within a CD3 gate (numbers inside quadrants represent the frequency of cells), and graphs of the frequency of CD4 and CD8 T-cells. B. Representative images of CD4 and CD8 immunohistochemistry staining of sigmoid biopsy sections from HIV-2-infected and seronegative individuals, and graphs of CD4 and CD8 counts in the HIV-2 and seronegative cohorts. Groups were compared using Mann-Whitney U test, and *P* values < 0.05 are shown.

Mucosal HIV-1 control is thought to depend upon local CD8 T-cell responses[33]. Of note, the HIV-2 cohort featured a relative increase in the frequency of mucosal CD8 T-cells (Fig. 1), and a significant expansion of those able to produce IFNγ (Fig. 2). Moreover, there were no alterations in the capacity of CD8 T-cells to produce other cytokines [% of IL-17 producing CD8 T-cells: 1.18 (0.6-2.0) in HIV-2 vs 0.8(0.5-3.1) in seronegatives, *P* = 0.87; and % of IL-22 producing CD8 T-cells 1.5 (0.9-2.6) vs 1 (0.5-1.8), *P* = 0.44].

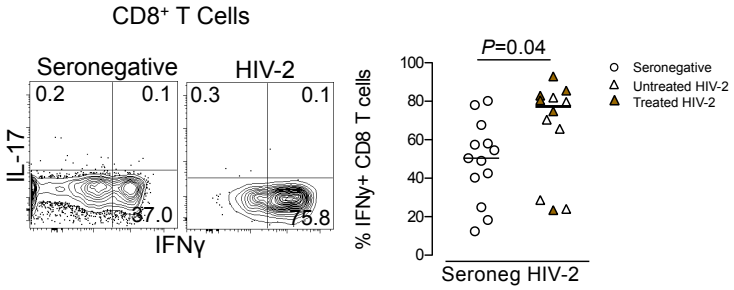

Figure 2. IFN-y-producing CD8 T-cells at the gut mucosa. Representative dot plots of the analysis of IFN-y and IL-17 production by total CD8 T-cells analyzed in cell suspensions obtained from sigmoid biopsies of HIV-2-infected and seronegative individuals with the right-hand graph showing the relative proportion of IFN-y-producing cells within CD8 T-cells in the two cohorts. Groups were compared using Mann-Whitney U test, and the *P* value is shown.

Our HIV-2 cohort included six untreated and seven patients under ART for a median of three years (Table 1). We found no differences in mucosal CD4 T-cell numbers between the treated and untreated HIV-2-infected individuals [483 (154-660) in 6 untreated vs 195 (55-616) in 7 treated, p= 0.47], or in any of the other parameters evaluated (data not shown).

Next, we investigated the relative representation of the different CD4 T-cell subsets based on their ability to produce cytokines and on the expression of regulatory T-cell (Treg) marker FoxP3.

It is known that IL-17 and IL-22 producing CD4 T-cells are particularly relevant for mucosal homeostasis[34, 35], and are preferentially depleted in HIV-1 infection.[11, 36] We found that HIV-2-infected individuals did not feature a depletion of these populations, with no difference in the frequency of total IL-17 producing or IL-22 producing CD4+ T-cells between the HIV-2 and seronegative cohorts (Fig. 3A).

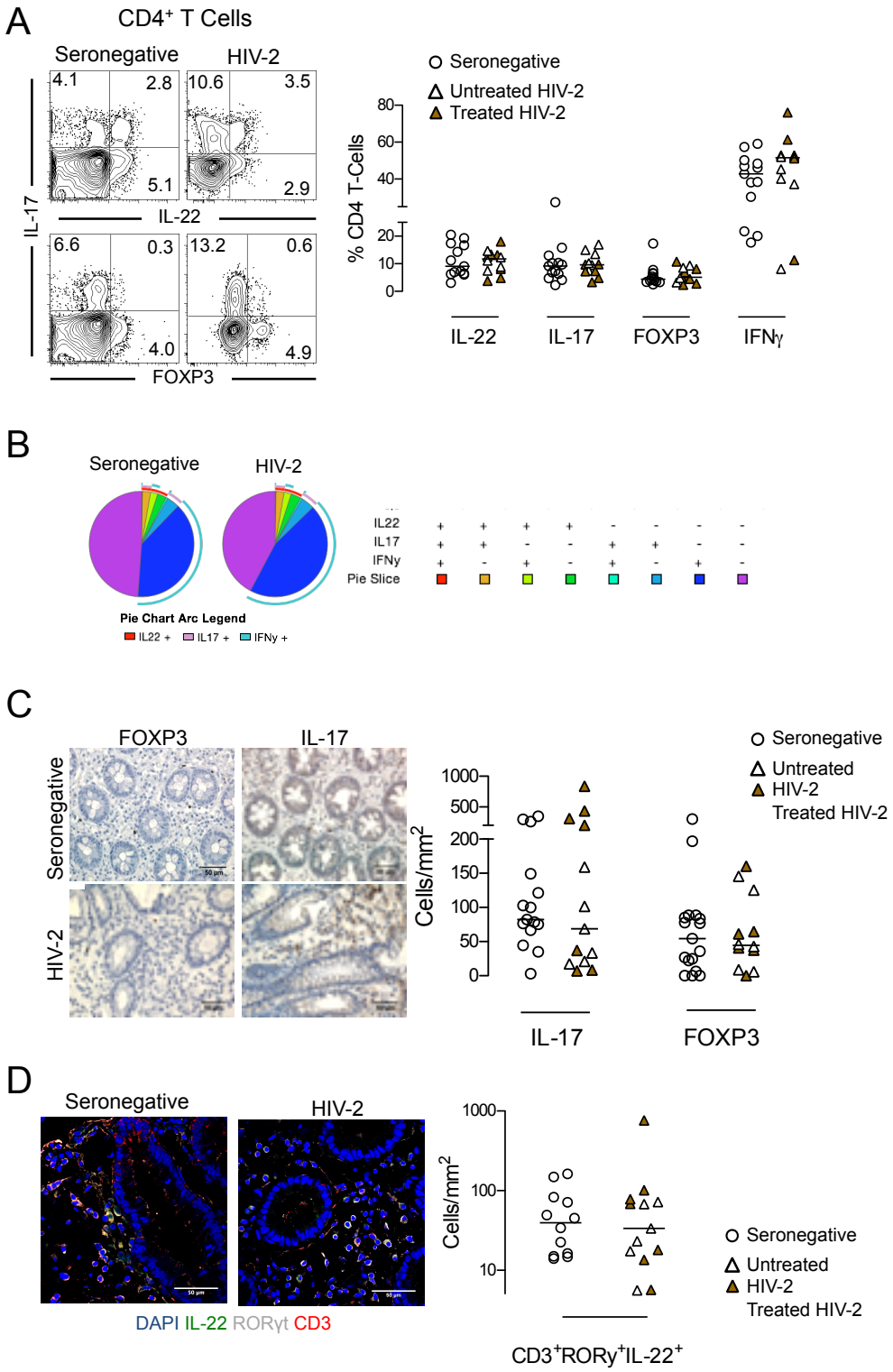

Figure 3. Gut mucosa CD4 T-cell subsets. A. Representative dot plots of IL-17, IL-22 and FOXP3 statining gated within CD4 T-cells isolated from sigmoid biopsies of HIV-2-infected and seronegative individuals, with graphs on the right showing the frequency of IL-17-, IL-22-, and IFNγ-producing cells, as well as FOXP3+ cells within total sigmoid CD4 cells in the HIV-2 and seronegative cohorts. B. Distribution of IL-17, IL-22 and IFNγ production within mucosal CD4 T-cells, determined using SPICE software. C. Representative immunohistochemistry images of IL-17 and FOXP3 cells in sections of sigmoid biopsy from HIV-2-infected and seronegative individuals, with graphs on the right showing the number of

positive cells in the HIV-2 and seronegative cohorts. D. Representative image of triple immunofluorescence staining using CD3, RORγ and IL-22, in sections of sigmoid biopsy from HIV-2-infected and seronegative individuals, with right-hand graph showing numbers of triple positive cells in the HIV-2 and seronegative cohorts. Groups were compared using Mann-Whitney U test, and *P* values < 0.05 are shown.

Furthermore, we observed no differences between the two cohorts in terms of the frequency of CD4 T-cells producing IL-17 alone [3.8(2.7-8.3)% in HIV-2 vs 3.6(3.0-5.7)% in seronegative; p=0.95], IL-22 alone [2.9(1.8-4.7)% in HIV-2 vs 2.8(2.1-5.3)% in seronegative; p=0.84], and both IL-17 and IL-22 [3.0(1.5-4.2) % in HIV-2 vs 2.8 (2.0-6.1)% in seronegative; *P* = 0.37].

The frequency of CD4 T-cells able to produce IFNγ was similar in HIV-2-infected and seronegative individuals (Fig. 3A).
Importantly, the distribution of the subsets able to produce one, two or the three cytokines (IL-17, IL-22 and IFNγ), was comparable in HIV-2-infected to seronegative individuals (Fig. 3B).

In addition, we confirmed that the number of IL-17$^+$ cells, as determined by immunohistochemistry (Fig. 3C), or of IL-22$^+$RORγ$^+$CD3$^+$ cells,as evaluated by immunofluorescence staining (Fig. 3D), were not different in HIV-2-infected and seronegative individuals.

The anti-inflammatory environment of the mucosa is, in part, maintained by FOXP3 expressing Tregs, known to expand during HIV-1 infection[12]. The HIV-2-infected individuals featured both similar numbers of FOXP3$^+$ cells, and frequencies of FOXP3+ expression within CD4 T-cells to seronegatives (Figs. 3A and 3C).

Of note, there was no correlation between the number or frequency of CD4 T-cells in the mucosa and peripheral blood (r=0.377, p= 0.31 and r=-0.27, p=0.5, respectively). There was also no association between mucosal CD4 T-cells and expression of immune activation markers in circulating CD4 T-cells (r = -0.206; p = 0.38), CD8 T-cells (r = -0.226; p = 0.34), or monocytes (r = -0.03; p = 0.91). As previously described the levels of immune activation were higher in black HIV-2-

infected individuals both for CD4 T-cells (% of HLA-DR+CD38+ within memory CD4 T-cells: 15.9(8.5-16.6) in blacks vs 4.6(3.3-6.3) in whites, p=0.003) and CD8 T-cells (% of HLA-DR+CD38+ within memory CD8 T-cells: 47.5(47.2-55.7) in blacks vs 12.0(9.0-26.5) in whites, p=0.006). However, this did not bias the results regarding immune activation as assessed by multiple regression analysis that included HIV group and race as co-variables (for the expression of HLA-DR and CD38 by CD4 T-cells: HIV-2 - coefficient = 1.82, p= 0.02 and black race - coefficient = 6.3 $p < 0.01$; for the expression of HLA-DR and CD38 by CD8 T-cells: HIV-2: coefficient = 8.3, p= 0.03 and black race: coefficient = 22.9, p = 0.01), despite the unbalanced distribution of race between the HIV-2 and seronegative groups (Table 1). This was also the case for all other parameters assessed in this study.

In summary, we found that HIV-2 infection was associated with preservation of sigmoid mucosa CD4 T-cells per se, as well as preservation of those subsets of particular relevance for gut mucosal homeostasis.

Maintenance of the Innate Lymphoid Cell compartment in HIV-2 infection

Mucosal epithelial homeostasis also depends upon IL-22 production by a subset of innate lymphoid cells, the ILC3, characterized by their lack of expression of lineage markers (CD3, CD19, CD14, CD16 and CD123), and expression of cKit and NKp44. We evaluated the sigmoid biopsies from the 13 HIV-2 individuals using these criteria, and found similar frequencies of these cells within total $CD45^+$ cells in both cohorts (Fig. 4A). Absolute numbers of these cells, defined as $CD3^-$RORγ+IL-22+ cells using immunofluorescence staining (Fig. 4B), also did not significantly differ between HIV-2 infected and seronegative individuals.

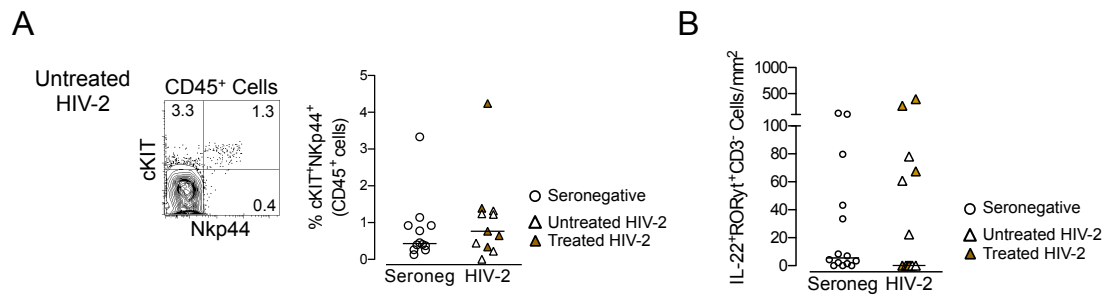

Figure 4. Gut mucosa type 3 innate lymphoid cells. A. Representative dot plot of cKit and NKp44 analysis within CD45+/lineage negative cells of an untreated HIV-2-infected individual (right) and graph showing the frequency of cKIT+Nkp44+ cells within a CD45+/lineage negative gate in HIV-2 and seronegative cohorts. B. Number of mucosa IL-22+RORγ+CD3- cells evaluated by triple immunofluorescence staining of sigmoid biopsy sections from HIV-2-infected and seronegative individuals. Groups were compared using Mann-Whitney U test, and *P* values <0.05 are shown.

As ILC3 constitute the main source of IL-22 within the CD3 negative compartment, we assessed the frequency of IL-22 producing cells within a $CD3^-$ gate instead of within cKit+NKp44+ cells to avoid possible errors associated with low event numbers. We found no differences between HIV-2 infected individuals and seronegatives regarding the frequency of IL-22+ cells [% IL-22 within CD3 negative cells: 6.1(2.9-7.5) in HIV-2 vs 2.6 (0.8-9.0) in seronegatives, $P = 0.13$].

ILC3 can also be defined by their ability to produce IL-17. Again, we found no difference in the frequency of $CD3^-$, IL-17 producing cells [% IL-17 within CD3 negative cells: 2.5(2.1-3.5) in HIV-2 vs 2.6 (0.8-9.0) in seronegatives, $P = 0.14$].

Overall, mucosal ILC3 appear to be maintained in HIV-2 infected individuals, as well as retaining their capacity to produce the homeostatic cytokines IL-17 and IL-22 by the innate compartment.

Gut mucosa integrity in HIV-2 infected individuals

We next asked whether the apparently normal frequency and function of the mucosal CD4 T-cells and ILC3 was translated into maintenance of epithelial integrity.

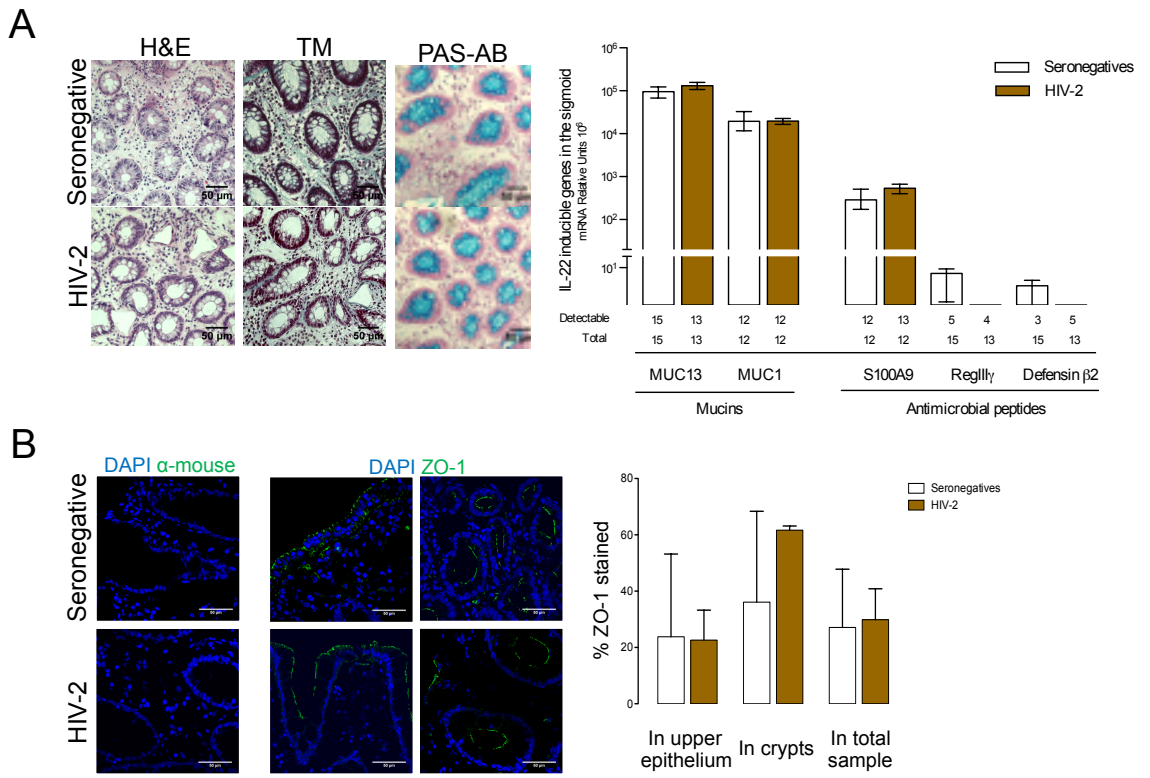

Figure 5. Gut mucosa structure and markers of epithelial integrity. A. Representative Haematoxylin and eosin (H&E), Masson's trichrome, and PAS-AB staining of sigmoid biopsies from HIV-2-infected and seronegative individuals, used to score the histological alterations: mean value of 1.25 for HIV-2+ (n=12) and 1.4 for seronegatives (n=15); Bars = 50µm. B. mRNA levels of IL-22-induced molecules; mucins and antimicrobial molecules induced by IL-22, in biopsies from HIV-1 and control cohorts. Numbers indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*, with bars representing medians and interquartile range. Comparisons were made using Mann-Whitney U test; and no significant differences were found. C. Illustrative immunofluorescence staining of ZO-1 in HIV-2-infected and seronegative individuals, with graphs showing the quantified proportion of epithelial area staining positive for ZO-1 in HIV-2 and seronegative groups; Bars = 50µm.

We found a relatively preserved structure of gut mucosa in HIV-2-infected individuals (Fig. 5A). A blind assessment by a trained pathologist revealed no significant difference in cellular infiltrate within the colon lamina propria (neutrophils, lymphocytes and eosinophils), resulting in a comparable inflammatory score as compared to seronegatives (Fig. 5A). Additionally, collagen deposition was assessed, and we observed no evidence of increased collagen deposition in any of the samples from HIV-2 infected individuals (Fig. 5A). Moreover, the bulk mucin production, evaluated though PAS-AB staining, showed no qualitative inter-cohort

differences (Fig. 5A). We also measured gene expression levels of mucins and antimicrobial peptides and did not find any difference between the cohorts (Fig. 5B),

The prevention of microbial translocation and increased gut permeability to microbial molecules requires intact epithelial tight junctions, structures that are known to be disrupted in the context of HIV-1 infection[13]. We assessed the levels of ZO-1, a tight junction protein, by immunofluorescence and found it was similarly expressed in samples from HIV-2-infected and seronegative individuals (Fig. 5C).

Our data regarding the preserved histological structure, along with epithelial tight junctions, suggest that of mucosal integrity is maintained during chronic HIV-2 infection.

Evidence of HIV-2 viral replication at the gut mucosa

Next we investigated whether a lack of local viral replication could underlie the apparent preservation of the gut mucosal environment, in terms of CD4 T-cells, ILC3 and mucosal integrity in HIV-2-infected individuals.

To do this we assessed viral replication in sections of gut mucosa using Gag immunohistochemistry staining for the HIV-2 Gag protein. As illustrated in Fig. 6, we were able to detect HIV-2 Gag positive cells in 5 out of 13 samples analyzed irrespective of antiretroviral treatment (2 out of 6 untreated and 3 out of 7 ART-treated patients were positive), despite the undetectable viremia documented in all individuals. A section of in-vitro HIV-2 infected TOC was processed identically to the gut biopsy-derived sections and stained with anti-Gag antibody, to serve as a positive control for Gag-staining (Fig 6A).

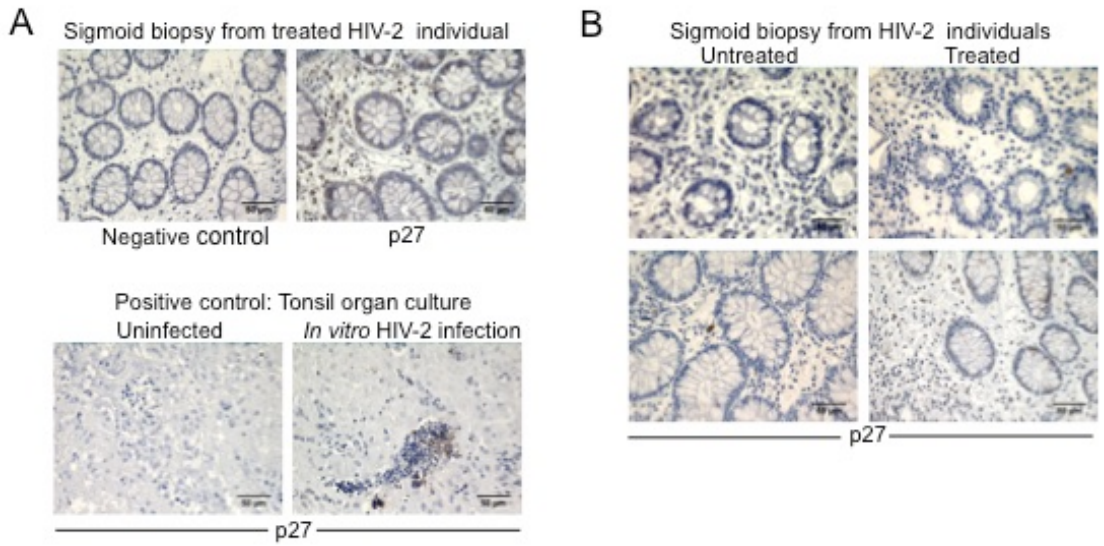

Figure 6. **Gut mucosa expression of HIV-2 gag.** A. The left-hand upper panel shows a negative control: a section of sigmoid biopsy from an ART-treated HIV-2-infected individual stained with secondary antibody alone. The right-hand upper panel shows a section from the same biopsy stained with anti-Gag (p27). The lower panels show Gag (p27) staining of an uninfected TOC (left-hand panel) and a TOC in-vitro infected with HIV-2 ROD for seven days (right-hand panel), the latter serving as a positive control for Gag staining. Bars = 50μm. B. Images of Gag (p27) immunohistochemistry staining of sigmoid biopsy sections from four different HIV-2-infected individuals (2 ART-treated and 2 untreated patients). Bars = 50μm.

Importantly, CD4 T-cell number and subset distribution did not differ in those patients with detectable Gag protein as compared to those without.

Thus, we were able to provide evidence of viral replication within the gut microenvironment, concomitantly with maintenance of mucosal homeostasis during chronic HIV-2 infection.

Mechanisms underlying gut homeostasis in HIV-2 infection

In order to evaluate the factors contributing to gut homeostasis during chronic HIV-2 infection, we quantified the transcriptional levels of cytokines and molecules known to be involved in the differentiation and maintenance of functional CD4 T-cells, particularly Th17, Th22 and Treg subsets, as well as ILC3.

We found comparable levels of expression of these molecules, which included IL-6, IL-23, and TGFβ, in the mucosa of HIV-2-infected and seronegative individuals (Fig. 7). Importantly, the relative amounts of the key anti- and pro-inflammatory cytokines, IL-10 and IL-1β were also similar in both cohorts.

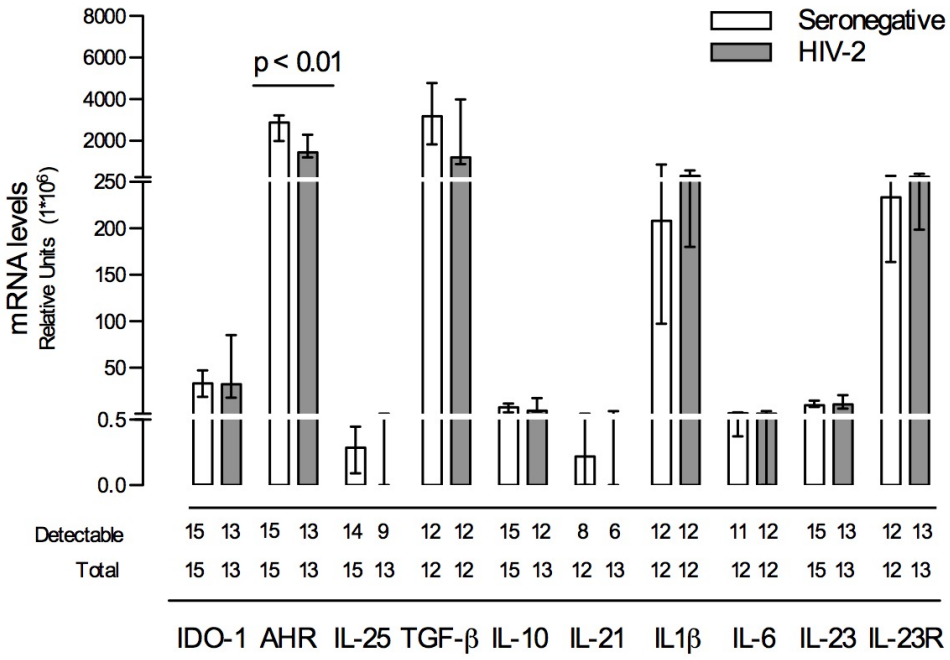

Figure 7. Expression of molecules relevant for gut homeostasis. *IDO1, AHR, IL-25, TGFβ, IL-10, IL-21, IL-1β, IL-6, IL-23,* and *IL-23R* mRNA levels in total RNA extracted from sigmoid biopsies of HIV-2-infected and seronegative individuals. Numbers below indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*. Bars represent medians and interquartile range. Comparisons were made using Mann-Whitney U test; and *P* values < 0.05 are shown.

Expression of IDO-1, the gene encoding the IDO enzyme Indoleamine-pyrrole 2,3-dioxygenase (IDO) that metabolizes Tryptophan, a process linked with lymphocyte suppression, was similar in both cohorts. This contrasts with the deregulated production of pro- and anti-inflammatory factors that occurs during HIV-1 infection and which is thought to contribute to the overall loss of mucosal CD4 T-cells, particularly the Th17 and Th22 subsets[9, 37].

On the other hand expression of the Aryl-Hydrocarbon Recptor (AHR) gene was reduced in HIV-2 infected compared to seronegative individuals. This receptor is known to be expressed on ILC3 subsets, and has been shown to be important for their survival, proliferation and function[38, 39]. However, its regulation is complex[39], and it is possible that reduced gene expression levels are related to increased signaling via the receptor.

Notably, we found a significant increase in CCL20 and CCL28 expression in the mucosa of HIV-2 infected individuals (Fig, 8A). These chemokines have been implicated in the recruitment of T-cells to the gut mucosa[40], specifically Th17 that express CCR6, the receptor for CCL20, and Th22 that express CCR10, the receptor for CCL28.

Of note, despite the lack of over-expression of key pro-inflammatory cytokines, we found HIV-2-infected individuals had significantly higher levels of GM-CSF transcription than their seronegative counterparts (Fig, 8A). Moreover, there was a direct correlation between the levels of GM-CSF and the expression of both CCL20 and CCL28 (Fig. 8B), suggesting that GM-CSF may help orchestrate this chemokine response.

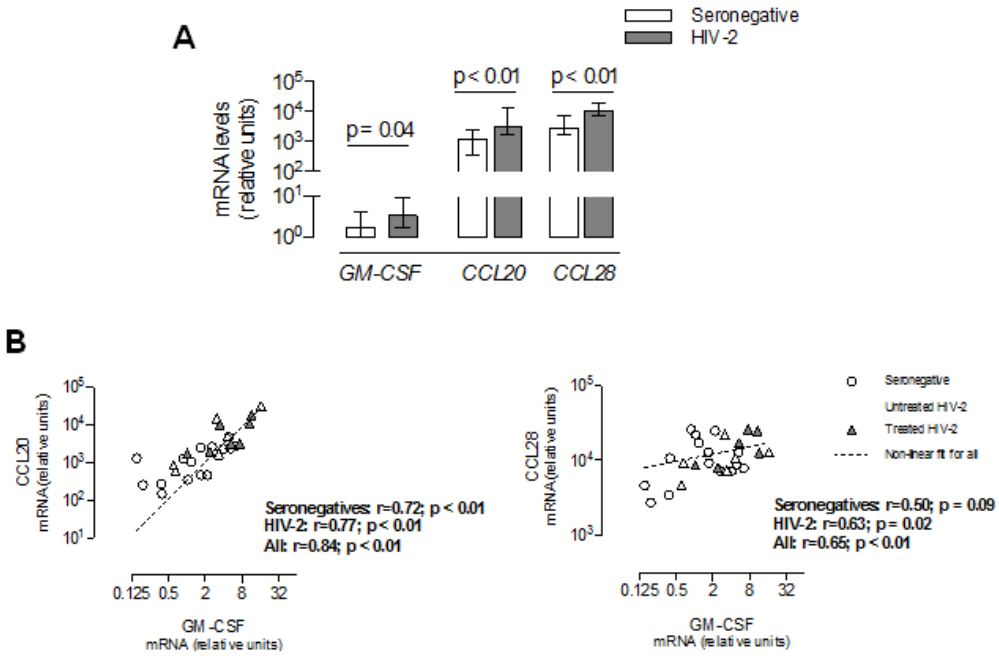

Figure 8. Expression of chemokines implicated in CD4 T-cell recruitment to the gut mucosa. A. *GM-CSF*, *CCL2*0 and *CCL28* mRNA levels in total RNA extracted from sigmoid biopsies of HIV-2-infected and seronegative individuals. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*. Bars represent medians and interquartile range. Comparisons were made using Mann-Whitney U test; and and *P* values < 0.05 are shown. B. Association between the levels of transcription of *CCL2*0 (left graph) and *CCL28* (right graph) with *GM-CSF* mRNA. Spearman correlation was used to calculate r coefficient and *P* values.

Overall, our findings suggest that increased cell recruitment may play a role in maintaining CD4 T-cells in the colon lamina propria of HIV-2-infected individuals.

Discussion

This study reveals, for the first time, evidence of persevered gut mucosal homeostasis in HIV-2 infection, providing a possible explanation for the slower rate of increase in immune activation associated with this natural model of attenuated HIV disease. We found that individuals with chronic HIV-2-infection featured: 1) lack of sigmoid CD4 T-cell depletion; 2) maintained Th17 and Th22 compartments in the absence of increased regulatory T-cells; 3) preserved ILC3 cells, and innate production of IL-22 and IL-17; and 4) normal mucosal structure and epithelial integrity; assessed by immunohistochemistry, expression of relevant epithelial genes and the tight junction protein ZO-1. Moreover, we showed that even those individuals with local HIV-2 replication featured no apparent mucosal CD4 depletion or gut disruption. Our data also suggest that mucosal CD4 T-cells are maintained during chronic HIV-2 infection through increased cell recruitment to the colon, as indicated by the elevated local production of relevant chemokines.

Gut disruption is thought to underlie HIV-1 immunopathogenesis, and to contribute to the chronic state of immune activation that persists in treated individuals [8, 41]. Of note, the seronegative cohort used in the current study was also used in our study of gut mucosa of treated HIV-1 individuals [24] that demonstrated significant CD4 T-cell depletion, particularly Th22 cells, despite prolonged, otherwise effective ART. This continued dysregulation of gut CD4 T-cell homeostasis may explain in part the persistent immune activation seen in ART-treated HIV-1 infection that has been linked to increased morbidity and mortality in these individuals[42].

In HIV-1 infection the hyper-immune activation that determines progression to AIDS has been intimately linked to the chronic disruption of gut mucosa and consequent translocation of microbial molecules[8,14,16]. Our HIV-2 cohort featured increased levels of systemic immune activation, assessed both in T-cells and monocytes, despite the evidence of a preserved gut mucosa homeostasis supporting the contribution of other factors to the hyper-activated state. Notwithstanding this, it is plausible that the slower progression towards AIDS that defines HIV-2 infection may be at least partly ascribed to the absence of the critical contribution of a leaky

gut to the pan-immune activation. HIV-1 infection is characterised by massive depletion of gut CD4 T-cells that occurs during acute infection[9] and persists throughout its course. Of note, there are no reports detailing the impact of HIV-2 acute infection on the gut. However, we have shown marked mucosal CD4 depletion and gut disruption in an 18 year old likely infected with HIV-2 since birth[43]. This suggests that eventually HIV-2 infection can result in a similar dysergulation of mucosal homeostasis, albeit over a much greater period of time than in HIV-1 infection and /or upon acquisition early in life with an immature immune system.

Importantly, we did not find any change in sigmoid epithelial structure, particularly with regard to tight junction protein expression, supporting, in conjunction with our previous data indicating a lack of increase of LPS[21], absence of a leaky gut in HIV-2 infection. A previous study, based in Guinea-Bisau, did observe increased LPS levels in the context of HIV-2 infection, but only in individuals who were classified as reaching AIDS (< 200CD4 T-cells/μl)[22].

Gut mucosal homeostasis is known to rely, together with other factors, on lamina propria CD4 T-cells, particularly Th17 and Th22 that maintain epithelial integrity and control commensal flora[35], and Tregs that maintain a tolerant environment compatible with the absorption of nutrients[44]. In our study of HIV-2 infection we did not find any evidence of perturbation in this equilibrium, in contrast to what has been shown for HIV-1 infected individuals, even those receiving long-term treatment, as we have previously reported[24]. This data regarding gut Th17 and Th22 cells in HIV-2 infection is important given the preferential impact that HIV-1 infection has on IL-17-[10] and IL-22-[11] producing cells in the gut, and the concomitant increase in mucosal Tregs[12], that occurs even from the earliest stages of infection.

Additionally, ILC3, through their production of IL-22[45] and crosstalk with CD4 T-cells[19, 46], have been shown to be fundamental to mucosal homeostasis. Our HIV-2 cohort also provided evidence that the numbers and frequency of these cells are maintained in the context of infection, as well as featuring a maintained capacity of the CD3 negative compartment to produce IL-17 and IL-22. Data from SIV studies suggest that the ILC compartment is severely depleted during all phases of infection,

occurring rapidly in the acute stage and persisting into throughout the disease [47, 48]. We have shown that the ILC compartment appears to be normal in long-term ART treated HIV-1 individuals, and may help compensate for the observed loss of Th22 cells in these individuals [24].

Importantly, we found evidence of viral replication in the gut mucosa of 5 patients, despite the lack of detectable plasma viremia in all patients. Thus this study provides additional evidence for ongoing viral replication in HIV-2-infected individuals despite the control of circulating plasma viral load as we and others have previously reported[5, 49].

Of note, viral replication was demonstrated in 2 out of the 7 HIV-2 ART-treated individuals, and overall ART did not segregate patients in terms of any of the parameters evaluated. This adds to the limited information about the impact of ART on HIV-2 induced disease[50], and supports the idea that therapies designed for HIV-1 might be less efficient in the context of HIV infection[49].

In agreement with ongoing mucosal viral replication we found an expansion of mucosal IFN-γ-producing CD8 T-cells. We were unable to study the specificity of these CD8 T-cells due to cell number constraints, but it is plausible to speculate that they may be involved in the local control viral replication, as suggested by previous gut studies in HIV-1[33] and in studies of peripheral blood in HIV-2 infected individuals[51]. Mucosal containment of viral replication within the mucosa might also be more effective in HIV-2 infection due to the absence of chronic disruption, in agreement with *in vitro* studies that showed that mucosal HIV-1 viral replication is facilitated by the access of microbial molecules to the lamina propria[52].

In spite of viral replication, the gut mucosa environment was apparently unaltered, particularly with respect to the expression of pro- and anti-inflammatory molecules, contrasting to what has been described for HIV-1 infection[14, 37]. Of relevance, molecules involved in IL-22 and IL-17 induction in T-cells and ILC3, namely IL-6 and IL-23[53, 54], were normally expressed. Also, factors involved in controlling mucosal inflammation, such as IL-10, were unchanged.

Of note, we did not find increased levels of TGFβ, which might explain the lack of collagen deposition. This may in turn help preserve CD4 T-cells, since, in HIV-1 infection, an association between TGFβ expression, collagen deposition and CD4 depletion has been demonstrated.[55, 56]

Interestingly, we found an increase in the expression of CCL20 and CCL28, that positively correlated with increased of GM-CSF levels. This raises the hypothesis that in HIV-2 infection mucosal cell numbers are maintained by an increased recruitment to the lamina propria. Due to the limited amount of tissue it was not possible to ascertain the origin of this increased production. Studies in HIV-1 infected individuals have implicated decreased CCL20, as well as preferential infection of CCR6+, Th17[57, 58], and CCR10+, Th22[11] cells in mucosa CD4 T-cell depletion and lack of recovery upon ART[25]. Data suggest that GM-CSF, together with IL-22, is produced at high levels by the CD90+ subset of Th17 cells, preferentially depleted in HIV-1 infection[59]. More recently, GM-CSF production by ILC3 has been linked to the promotion of Intestinal homeostasis[60]. Thus it is possible that increased levels of GM-CSF we observed are indicative of maintained gut homeostasis. Our finding of increased CCL20 expression in the mucosa without a concomitant increase in Th17 could indicate that some degree of loss of these cells is occurring, but unlike in HIV-1 infection[25], the mechanisms involved in their replenishment are preserved, even after many years of a persistent chronic infection.

Conclusion

Overall, we revealed for the first time that chronic HIV-2 infection is associated with a lack of CD4 T-cell depletion at the gut mucosa together with preserved epithelial integrity. Our data provide a possible explanation for the slow increment in the levels of immune activation and the relatively benign course of HIV-2 disease. Moreover, we found that the mucosal CD4 T-cell homeostasis is likely to result from a local response with over-expression of critical homeostatic chemokines, which have implications for the design of therapies aimed at acheiving gut recovery in HIV-1 infection.

Author contribution: Performed research: S.M.F., A.R.P., C.F., P.M. and RB; Clinical data and sample collection: S.M.F., L.C., E.V., J.P. and P.P.; Designed the study, analysed data and wrote the paper: S.M.F., R.B., H.V.F. and A.E.S.

Acknowledgments

We would like to thank the individuals that participated in the study, as well as the physicians for their follow-up and collection of clinical data, particularly Joana Fernandes, Manuela Doroana, Sérgio E. Paulo, Nuno Janeiro, and Francisco Antunes from the Department of Infectious Diseases of Hospital de Santa Maria, Lisbon; the collaboration of the Gastroenterology Departments of Hospital de Santa Maria, Lisbon, and Hospital de S. Bernardo, Setúbal, for the endoscopic studies; Vânia Silva for the technical assistance in molecular biology; and Helena Nunes-Cabaço, Iris Caramalho, and Rui M.M. Victorino for the critical discussion of the manuscript.

Conflict of Interests: The authors have no conflict of interest to report.

Funding: This work was supported by the Fundação para a Ciência e Tecnologia (FCT), and the Programa Operacional Ciência e Inovação 2010 (EXPL/DTP-PIC/0854/2013 to RBF, and PIC/IC/82712/2007 to AES), as well as Fundação Calouste Gulbenkian (to AES). SMF, ARP and RBF received FCT scholarships.

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Supplementary data

Supplementary Table 1. Monoclonal Antibodies used in Flow Cytometry

Antibody	Fluorochrome	Clone	Source
IL-17	FITC	eBIO54DEC17	eBioscience[a]
IL-22	PE	22URTI	eBioscience
FOXP3	V450	PCH101	eBioscience
CD117 (cKIT)	PerCP	104D2	eBioscience
CD45	V450	2D1	eBioscience
CD45RO	APC-Cy7	UCHL1	eBioscience
CD3	V500	UCHT1	eBioscience
CD4	APC	RPA-T4	eBioscience
CD8	PerCP	RPA-T8	eBioscience
CD14	PE-Cy7	61D3	eBioscience
CD19	APC-efluor780	HIB19	eBioscience
CD16	FITC	eBioCB16	eBioscience
CD38	PE	HB7	eBioscience
HLA-DR	PerCP-efluor	L243	eBioscience
IFNy	PE-Cy7	4S.B3	Biolegend[b]
CD123	V450	6H6	Biolegend
Nkp44	APC	243415	R&D[c]
CCR7	FITC	150503	R&D
CD25	PE-Cy7	M-A251	BD Bioscience[d]
CD45RO	APC-H7	UCHL1	BD Bioscience
CD56	PE	My31	BD Bioscience
CD11c	APC	B-Ly6	BD Bioscience
CD3	V450	UCHT1	BD Bioscience
CD4	V500	RPA-T4	BD Bioscience

[a]eBioscience, San Diego, CA; [b]Biolegend, San Diego, CA; [c]R&D, Minnneapolis, MN, USA; [d]BD Bioscience, San Jose, CA.

Supplementary Table 2. Antibodies used in Immunochemistry

Antibody	Clone	Source
CD4	4B12	Leica Biosystems[a]
CD8	1A5	Leica Biosystems
FOXP3	236/E7	Leica Biosystems
IL17	polyclonal	Santa Cruz
P27	monoclonal	NIAIDc

[a]Leica Biosystems, Wetzlar, Germany; [b]Santa Cruz Biotechnology, Dallas, USA.[c]AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from Dr. P. Szawlowski

Supplementary Table 3. Antibodies used in Immunofluorescence Staining

	Antibody	Source
Primary antibodies	Rabbit anti-RORγt	Abcam[a]
	Goat anti-IL-22	Santa Cruz Biotechnology[b]
	Rat anti-CD3	Acris[c]
	Mouse anti-ZO-1	BD Biosciences[d]
	Alexa Fluor 488 Anti-Goat	Molecular Probes[e]
Secondary antibodies	Alexa Fluor 488 Anti-Mouse	Molecular Probes
	Alexa Fluor 546 Anti-Rabbit	Molecular Probes
	Alexa Fluor 647 Anti-Rat	Molecular Probes

[a]Abcam, Cambridge, UK; [b]Santa Cruz Biotechnology, Dallas, USA; [c]Acris, Herford, Germany; [d]BD Bioscience, San Jose, CA; [e]Molecular Probes, Life Technologies, Carlsbad, USA.

CHAPTER 4. CONCLUSIONS AND FUTURE PERSPECTIVES

Gut mucosal homeostasis is fundamental for human health, not only because it secures nutrient digestion and absorption, but because it influences systemic immune responses, guaranteeing a balanced relationship with the external environment[1, 2].

HIV-1 infection is associated with loss of gut mucosal integrity during all stages of infection. HIV-1-associated gut disturbances are characterized by depletion of CD4 T-cell that occurs early and persists throughout infection[3-5], and altered proportions of CD4 T-cell subsets, particularly IL-17 producing cells (Th17)[6,7]. This ultimately results in gut epithelial disruption and passage of microbial products into the circulation, which in turn drives disease progression[8, 9].

Antiretroviral therapy (ART) has completely changed the natural history of HIV-1 infection, and nowadays the survival of HIV-1 infected patients is, in some cohorts at least, comparable to seronegative individuals, particularly for those individuals who started antiretroviral drugs before development of significant immunodeficiency[10]. Despite this achievement, ART-treated HIV-1 infected individuals still feature an increased morbidity that has been associated with increase immune activation[11], possibly resulting from a leaky gut[12]. In fact, the degree to which gut mucosa integrity is re-established in HIV-1 infected individuals under long term effective ART is still the issue of debate[13]. The currently accepted theory is that long-term treated HIV-1 infected individuals still feature increased gut permeability. However, different results were generated in distinct cohorts, and were dependent on the methods used to assess gut integrity[14-16].

IL-22 has recently been shown to be fundamental for both gut epithelial homeostasis and integrity[17, 18]. In our study of 17 long term treated HIV-1 individuals, we showed: 1) decreased IL-22 production by CD4 T-cells; 2) preservation of IL-22 production by the CD3 negative compartment and, importantly, a normal frequency of innate type 3 lymphoid cells (ILC3); 3) maintenance of epithelial integrity; 4) normal expression of genes dependent on IL-22 (Chapter 3.1.).

The apparent paradox between decreased IL-22 production by T-cells and unaltered gut homeostasis might be explained by the normal production of IL-22 by

ILC3. These results support the importance of innate sources of IL-22 in ensuring epithelial integrity in the context of a chronic retroviral infection.

The differing location of ILC3 and Th22 in the gut mucosa, as well as the amount of IL-22 produced by each cell type, might explain preserved IL-22 dependent mechanisms in the context of chronic reduction of Th22, although we did not find an increase in ILC3 in the lamina propria. Of interest, recent murine studies have shown that CD4 depletion in the mucosa results in increased ILC3 numbers and IL-22 production by these cells, as a compensatory mechanism to ensure mucosal homeostasis[19].

Another group recently showed a preferential depletion of IL-22 producing CD4 T-cells in both the acute and chronic phases of infection that apparently completely recovered upon prolonged ART-treatment[20]. These discrepant results may be partially explained by the differing ages of the HIV-1 individuals and the healthy cohort used as control in this study. Importantly, we showed a positive association between age and frequency of IL-22 producing CD4 T-cells in our cohort of seronegatives. Given that almost all our patients were recruited to the study because of colorectal cancer screening, our study cohorts were almost perfectly matched for age.

It is now currently accepted that starting ART late in HIV disease is associated with cumulative damage to the mucosa tissue, reducing the possibility of recovery[21]. It is worth mentioning that there is little evidence from human studies comparing recovery of gut homeostasis between individuals that start ART early vs. those that start later on in the course of disease[22]. A very recent human study, however, appears to point into that direction, showing a limited Th17 cell depletion upon very early treatment when compared with treatment in a later stage during infection[23]. Data from primate studies suggests that early antiretroviral treatment limits gut damage and favours complete recovery of gut homeostasis[24, 25]. Our cohort of HIV-1 treated patients had started treatment at a relatively advanced stage, as attested by a median of nadir CD4 counts of 339 cells/μL. Therefore, in our case, the apparent normalization of gut integrity cannot be attributed to early treatment.

On the other hand, the definition and strategies to evaluate mucosal homeostasis are still controversial. We performed a detailed analysis of mucosal integrity, by carefully assessing the epithelial structure, immunofluorescent staining of the tight junction protein ZO-1, and by quantifying the levels of gene expression of some of the most relevant mucins and antimicrobial products induced by IL-22. Nevertheless, our comprehensive analysis could not prove beyond doubt an absence of increased mucosal permeability. To illustrate the ongoing debate, a recent study found decreased levels of tight junction protein expression, as well as mRNA levels, in the distal colon of effectively treated HIV-1 individuals (with a median of 11 years treatment) which was not associated with micro or macro mucosal lesions[26]. It is particularly debatable how to best assess mucosal integrity in clinical practice, and its relevance for overall patient survival. In fact, in a recent case-control study, the plasma levels of I-FABP (a peptide released by damaged enterocytes) and zonulin were shown to correlate with mortality, but not T-cell activation, among HIV-1 patients treated for 12 months[27].

Ultimately, the definitive proof of the relevance of gut homeostasis to the overall survival and morbidity of HIV-1-infected individuals under ART will come from randomized controlled trials aimed at limiting microbial translocation in this setting. The first results, however, have not been promising. Rifaximine, a non-absorbed antibiotic that was shown to decrease endotoxinemia in chronic liver patients[28], had a minor effect on T-cell activation in a group of immune discordant HIV-1 treated individuals[29]. Moreover, studies addressing the effect of probiotics on several prognostic markers in treated HIV-1 infected individuals did not show clear benefits[30, 31].

Importantly, we are still looking for good markers of gut disruption. Their identification would facilitate the demonstration of the efficacy of therapies aiming mucosa homeostasis reconstitution. Systemic immune activation, however does not appear to be a good surrogate, although frequently used as a marker for gut disruption. On the other hand, lack of recovery of a particular mucosal CD4 T cell subset cannot be equated to loss of gut homeostasis and/or integrity, as we showed in our study.

Overall, mucosal homeostasis, including IL-22–mediated mechanisms essential for epithelial integrity, could mainly rely on ILC3, as suggested by our data on long-term-treated HIV-1–infected patients. Thus, the therapeutic targeting of ILCs may represent an important strategy to promote intestinal recovery.

HIV-2 infection is characterized by a relatively "benign" disease course, with little impact on mortality in infected adults[32]. In spite of the much better prognosis as compared to HIV-1 infection, and the typically low to undetectable viremia, HIV-2 infected individuals feature increased immune activation levels that determine CD4 T-cell loss, that leads to AIDS[33]. It is plausible that the factors underlying the immune activation in HIV-2-infected individuals differs to those described for HIV-1 infection, and we hypothesized that a major difference may be related to the contribution of gut mucosal disruption to this process.

We studied a cohort of 13 HIV-2 infected individuals, and showed no evidence of gut disruption, in contrast to HIV-1 infected. We used the equivalent strategy for evaluating mucosal integrity as for the HIV-1 study discussed above, and revealed 1) preserved CD4 cell counts; 2) maintained representation of CD4 T-cell subsets; 3) unaltered ILC3 compartment; 4) well-maintained epithelial integrity assessed by immunohistochemistry and immunofluorescence methods, as well as tight junction integrity and gut mucosa environment, with respect to expression of pro- and anti-inflammatory molecules (Chapter 3.2.1.).

Overall these results demonstrate for the first time that HIV-2 infection is associated with preservation of mucosal integrity. This is in agreement with previous data from our laboratory showing normal LPS levels in HIV-2 infected individuals[34]. The lack of mucosa disruption could limit viral replication on its own, since leakage of microbial peptides into the lamina propria contributes to local cell activation increasing the number of HIV cell targets[35]. On the other hand, the reduced microbial translocation could limit the range of potential targets of immune responses, that can occur in this context, possibly allowing a more focused and appropriate immune response[36].

Importantly, the cohort of seronegative individuals was the same used in our study of treated HIV-1 infected individuals, and the same investigators evaluated all the parameters, limiting the subjectivity of the analysis and increasing the consistency of these data. Additionally, all the individuals studied were age matched, since the major criterion of inclusion was clinical indication to perform colorectal cancer screening. Of note, in the HIV-2 infected cohort there were 5 black individuals in contrast to the seronegative group that did not include any. Although black individuals, as previously suggested[37, 38], have increased levels of immune activation, the exclusion of these individuals from the analysis did not change the results, which gives us confidence that race did not significantly bias our results.

Our HIV-2 cohort included 7 individuals under ART, but there were no significant differences between treated and untreated subjects. Of note, the treatment of HIV-2 infected patients was never formerly addressed in randomized controlled trials and it is claimed that therapies designed to target HIV-1 might be less effective in the context of HIV-2 infection, thereby limiting immune reconstitution[39].

Significantly, we found evidence of preserved mucosal integrity, as confirmed by analysis of mucosal structure and immunohistochemistry stainings, as well as ZO-1 quantification by immunofluorescence. This occurred in a scenario of normal mucosal CD4 T-cell frequency and counts. Importantly, there were also no imbalances between the relevant CD4 T-cell subsets: Th17, Th22, Th1 and Tregs, as well as overall cytokine production by CD4 T-cells. This equilibrium is known to be lost in HIV-1 infected individuals at all stages of HIV-1 infection[3, 5, 40], as well as in primate models of AIDS[41-44]. In contrast, in HIV-1 long-term non-progressors (LTNP) there is a preserved balance between the main mucosal CD4 T-cell subsets[45, 46], although HIV-1 still induces sufficient gut epithelial deregulation in this group to compromise long-term clinical outcome[47]. Moreover, natural primate hosts of SIV preserve mucosal integrity, and despite some transitory mucosal CD4 T-cell depletion during acute infection, CD4 T-cell numbers are maintained in conjunction with an apparent lack of epithelial damage[48].

Our observation of preserved mucosal CD4 T-cell counts was associated with an increase in expression of the chemokines CCL20 and CCL28. The former is involved in the migration of CCR6 expressing cells to the lamina propria, whereas the latter targets cells expressing CCR10. Interestingly CCR6 is mainly expressed on Th17[49] and CCR10 on Th22[50, 51]. Additionally, we found that GM-CSF levels were increased, and that they positively correlated with CCL20 and CCL28 levels. Several cell types in the gut mucosa: Th17, epithelial cells and stromal cells, produce GM-CSF[52, 53]. Of note, IL-17 induces a transcriptional program in epithelial cells that includes increased expression of IL-1, IL-6, TNFα, CXCL8, G-CSF and GM-CSF that helps in the recruitment of neutrophils. These are able to produce significant amount of CCL20, helping recruit more IL-17 producing cells[49]. Moreover, mucosal stromal cell production of GM-CSF, together with retinoic acid, in mice, imprints $CD103^+$ DC with the ability to produce retinoic acid[53], favouring the induction of Tregs[54]. In our study, we could not ascertain the source of GM-CSF, CCL20 or CCL28, or which cells contribute to their increase, due to the reduced amount of available tissue. Despite this limitation, these findings suggests that mucosal homeostasis in the context of HIV-2, a more benign disease, might rely on increased recruitment of T-cells to the gut mucosa, and maintenance of a normal balance between inflammatory and anti-inflammatory mechanisms.

Of relevance, our cohort of HIV-2 infected patients still feature increased levels of immune activation, assessed using both T-cell and monocyte markers. In HIV-1 infected individuals the levels of expression of HLADR and CD38 on T-cells, and particularly the frequency of $CD16^+$ monocytes have been associated with different parameters of gut disruption[8, 55, 56]. We did not find any correlation between gut mucosal parameters and peripheral immune activation levels assessed in CD4 T-cells or monocytes. Importantly, the ethical constraints and willingness of patients to submit to colonoscopies, despite the potential benefit of cancer screening[57], limited the numbers of patients recruited. Consequently the studies of human gut mucosa usually rely on small sample sizes[5, 21, 47, 58]. Thus, we cannot exclude that these small numbers are responsible for the lack of association between mucosal parameters and immune activation. Nevertheless, we did found increased systemic immune activation levels and normal mucosa parameters. Therefore, it is plausible to speculate that the

mechanisms driving immune activation in HIV-2 infected individuals are different from those in HIV-1, and may be more directly related to viral control[36]. Nevertheless, we remain unable to separate out the several components that potentially drive immune activation, and identify those that could define appropriate and inappropriate immune activation. It is also important that in HIV-2 infection, mechanisms that protect the immune system from the consequences of chronic immune activation are apparently operating, for example the concerted increase in the levels of expression of PD-1 and PD-L1 on T-cells, dendritic cells and monocytes, as reported by our lab[34, 59, 60]. Importantly, there are no studies addressing the clinical impact of this prolonged immune activation in HIV-2 infected individuals, specifically if it leads to increased cardiovascular risk and other non-AIDS related co-morbidities as in HIV-1 infected individuals[27, 61].

Of note, our study provided, for the first time, evidence of HIV-2 replication at gut lamina propria in spite of the undetectable plasma viremia. This is in agreement with previous evidence from our group reporting ongoing viral replication in HIV-2 infection despite the lack of detectable viremia, based on the quantification of *gag* mRNA in periphal CD4 T-cells[62]. The mechanisms underlying control of viremia are still unexplained. It is plausible that multiple factors contribute to the control of HIV-2 viral replication at the tissue level, including effective specific T-cell responses[63, 64] and/or potent neutralizing antibodies[65]. Interestingly, in LTNP, HIV-1 specific mucosal responses, particularly the HIV-specific CD8 T-cell response, are known to be relevant in guaranteeing viral control at this location[66, 67].

Overall, and despite evidence of HIV-2 replication in the gut mucosa, its integrity was preserved, possibly contributing to the more benign course of this infection. Expanding these studies to investigate the mechanism(s) involved in viral control in the gut mucosa, could possibly inform the design of new therapies for both HIV-1 and HIV-2 infection.

Of note, the results from our cross-sectional cohort study contrast with our longitudinal assessment of gut mucosal homeostasis in an 18 year old HIV-2 infected patient (Chapter 3.2.1.). Importantly, in this case viral infection occurred in early

childhood, and there was an AIDS diagnosis at the time of the first mucosal sampling. This unique patient featured increased levels of immune activation and detectable plasma viremia in association with evidence of mucosa disruption: altered mucosal architecture, decreased mucosal CD4 cell counts and increased plasma LPS levels. Importantly, antiretroviral treatment was able to reverse all these changes, and gut homeostasis was fully recovered, with the individual able to maintain, so far, mucosal integrity.

Although, these data might appear at first to be contradictory, there are several differences between this individual and the studied group of patients with chronic HIV-2 infection. The young age at which HIV-2 infection was acquired with an already immature immune system was likely a main determinant of the distinct outcome of this patient. A more rapid progression of HIV-1 disease towards AIDS upon acquisition at young age has been well documented[68], and although comparatively slower, this also holds true for HIV-2 related disease[69]. Moreover, this individual had an AIDS diagnosis. This contrast with the older HIV-2 cohort enrolled in the cross-sectional study that were submitted to colonoscopies for colorectal cancer screening, and featured an average CD4 counts of 490 cells/μL.

The first time point from our longitudinally studied patient was collected within the first three months of their arrival in Portugal from Guinea-Bissau, and showed increased plasma LPS levels. Of note, it has been previously reported that HIV-2 patients with AIDS recruited in Guinea-Bissau feature an increase in LPS levels, that, like in HIV-1 infection, was correlated with CD4 T-cell counts and viremia, suggesting that gut disruption could also contribute to HIV-2 pathogenesis[70]. However, in this context, it is plausible that chronic intestinal infestations and race might augment the state of hyper-immune activation, as previously mentioned[71]. On the other hand, the HIV-2 infected patients included in our cohort study were all living in Portugal for more than 5 years and showed evidence of mucosal integrity. These results were in line with a previous study published by our group performed in a distinct HIV-2 cohort that showed no significant increase in plasma LPS in HIV-2 infected patients, and, importantly, no correlation of LPS levels with monocyte activation or CD4 T-cell levels[34].

Of relevance, the longitudinal study of this case revealed a remarkable recovery of mucosal CD4 T-cell populations after 5 years of ART. The limited data available on HIV-2 treatment suggests a worse response to treatment[39, 72, 73] that might depend on several factors, namely the use of drugs not specifically designed to target HIV-2, and a longer course of infection prior to start of treatment. Notwithstanding this, our patient's recovery of mucosal CD4 T-cells suggests that HIV-2, contrary to HIV-1, induces a reversible mucosal lesion allowing for a full recovery of gut homeostasis with effective ART. All the collected gut samples in this case were paraffin embedded, precluding a quantification of mRNA of relevant transcripts, namely CCL20 and CCL28. If this hypothesis proves to be true, the further research into on how gut homeostasis is maintained in HIV-2 infection might help define cell targets to prevent HIV-1 disruption of gut mucosa.

Overall, our work has provided insights into how gut mucosal homeostasis might be maintained in the presence of persistent viral infection and consequent chronic inflammation. Given our results in HIV-1 treated individuals, it is plausible that ILC3 localization and their high level production of IL-22 compensate for the loss of IL-22 production from other sources, such as Th22. Additionally, an increase in the local production of relevant chemokines might be critical to ensure mucosal CD4 T-cell preservation through a balanced recruitment to face cell death at gut mucosa. Nevertheless, the mechanisms behind the control of inflammation at lamina propria despite evidence of ongoing viral replication in HIV-2 infection were not ascertained.

Altogether, this work has contributed to a better understanding of mucosal homeostasis in the context of two distinct chronic viral infections with different disease outcomes. We have shown recovery of epithelial integrity in long-term treated HIV-1 infected individuals, particularly IL-22 dependent mechanisms, that despite decreased Th22 were preserved, calling for further investigation into the role of ILC3 in the context of HIV infection. Additionally, we have reinforced the importance of the preservation of gut homeostasis in achieving a better clinical outcome in HIV disease, through the demonstration of lack of gut disruption in HIV-2 infected individuals.

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CHAPTER 5. ANNEXES

Annex 1 – Published articles included in the Thesis

Journal of Infectious Diseases Advance Access published April 4, 2014

MAJOR ARTICLE

Enteric Mucosa Integrity in the Presence of a Preserved Innate Interleukin 22 Compartment in HIV Type 1–Treated Individuals

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Background. Interleukin 22 (IL-22) is emerging as a key cytokine for gut epithelial homeostasis and mucosal repair. Gut disruption is a hallmark of human immunodeficiency virus (HIV) infection. Here, we investigated IL-22 production and gut mucosal integrity in HIV type 1 (HIV-1)–infected individuals receiving long-term antiretroviral therapy (ART).

Methods. Biopsy specimens from 37 individuals who underwent colonoscopy primarily for cancer screening and from 17 HIV-1–infected and 20 healthy age-matched controls were assessed.

Results. We found significant depletion of sigmoid IL-22–producing $CD4^+$ T cells (T-helper type 22 [Th22] cells) even after prolonged ART, contrasting with the apparently normal compartments of regulatory and interleukin 17 (IL-17)–producing $CD4^+$ T cells, as well as total mucosal $CD4^+$ T cells. Despite the preferential Th22 cell depletion, IL-22 production by innate lymphoid cells (ILCs) was similar to that observed in HIV-1–seronegative subjects, and transcription of genes encoding molecules relevant for IL-22 production (ie, *AHR*, *IL23*, *IL23R*, *IL1B*, *IL6*, and *TGFB1*) was preserved. Remarkably, levels of transcripts of IL-22–target genes (ie, *REG3G*, *DEFB4A*, *S100A9*, *MUC1*, and *MUC13*) were unaltered, suggesting an adequate production of antimicrobial peptides and mucins. In agreement, enteric epithelial architecture was fully preserved.

Conclusions. Despite the reduced Th22 cell subset, innate IL-22–mediated mechanisms, essential for sigmoid mucosa integrity, were fully operational in long-term–treated HIV-1–infected individuals. Our data highlight IL-22 production by ILCs as an important target for therapies aimed at facilitating human mucosal reconstitution.

Keywords. Gut Associated Lymphoid Tissue; HIV/AIDS; Mucosa Reconstitution; IL-22; Antiretroviral Therapy.

The gut mucosa, the body's largest interface with the external environment, provides the first microbial barrier. Furthermore, its interaction with the microbiome actively shapes the systemic immune system and determines human health [1]. Interleukin 22 (IL-22) has emerged as a key cytokine in these processes [2]. Signaling via the IL-22 receptor induces proliferative and antiapoptotic molecules in gut epithelial cells [3], which have been shown to be critical for epithelial homeostasis, particularly in the context of mucosal insults. Additionally, IL-22 signaling triggers epithelial production of antimicrobial peptides, which are relevant both for the containment of the gut microflora and the response to infections [4].

While the IL-22 receptor is essentially expressed by nonhematopoietic cells, specifically the gut epithelia, IL-22 itself is produced, under tight regulation, by hematopoietic cells [2]. Of note, high rates of epithelial proliferation due to deregulated IL-22 production have been associated with colon cancer [5]. In the context of epithelial lesions, shedding of microbial products

Received 23 November 2013; accepted 13 February 2014.

Presented in part at: Keystone Symposia—Immune Activation in HIV Infection: Basic Mechanisms and Clinical Implications, Breckenridge, Colorado, 3–8 April 2013. Poster 1035.

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The Journal of Infectious Diseases

DOI: 10.1093/infdis/jiu126

leads to the secretion of interleukin 23 by dendritic cells [6], which, in conjunction with interleukin 1β and interleukin 6, induces IL-22 production. On the other hand, interleukin 25 secretion by the intact/repaired epithelium is known to inhibit this process [7].

The main IL-22 producers in the gut mucosa are $CD4^+$ T cells (T-helper type 22 [Th22] cells) [8] and a subset of innate lymphoid cells (ILCs), named ILC3 [9]. It is currently unknown whether factors governing IL-22 production impact distinctly on the above 2 populations or whether these populations differentially contribute to the preservation of mucosal integrity [10].

ILC3 were initially described as participating in the development of gut-associated lymphoid tissue (GALT) [11] and were later shown to be critical for the response to tissue injury in adult life in the gut, thymus, and lymph nodes [12, 13]. In addition to their nonredundant contribution to the control of attaching-effacing enteric infections [14], ILC3 were recently reported to suppress $CD4^+$ T-cell activation in response to commensal bacteria [15], further emphasizing their fundamental role in intestinal homeostasis.

IL-22 is likely to be implicated in the pathogenesis of human immunodeficiency virus (HIV) infection and AIDS, given the profound disruption of gut mucosa typically observed throughout disease [16]. Acute HIV type 1 (HIV-1) infection leads to a massive loss of mucosal $CD4^+$ T cells, which persists into the chronic phase [17], and is inconsistently recovered upon long-term antiretroviral therapy (ART) [18]. Loss of epithelial integrity and high levels of circulating microbial products have been reported even in treated patients and are considered important contributors to HIV-associated pan-immune activation that underlies the pathogenesis of HIV infection and AIDS [19–21]. The heightened activation state of the immune system is also associated with an increased risk of noninfectious events in ART-treated individuals.

Most previous studies of the gut of HIV-1–infected patients focused on the loss of IL-17–producing cells [22]. However, given the overlap between the mucosal populations that produce IL-22 and/or interleukin 17 [10], it is possible that some of the reported disturbances were due to IL-22. Interestingly, although IL-22 was initially described as a Th17 cytokine, there are several factors supporting a unique identity for IL-22–producing cells [8, 10]. Production of these 2 cytokines is linked to expression of the transcriptional factor retinoic acid orphan receptor (ROR)γt and the homing receptor CCR6 [23]. However, aryl hydrocarbon receptor (AHR) ligands have a preferential impact on IL-22 production [24]. Additionally, transforming growth factor β (TGF-β) induces IL-17 secretion while inhibiting IL-22 production via c-Maf induction in $CD4^+$ T cells [25]. Nevertheless, concomitant production of IL-17 may balance positive and negative impacts of IL-22, as suggested in inflammatory bowel disease [26].

The limited data available suggest that mucosal IL-22 production is reduced during chronic HIV-1 [27] and simian immunodeficiency virus (SIV) infections [28]. However, mucosal distribution of enteric IL-22–producing cell populations in humans is still poorly defined. Furthermore, their relationship with the expression of genes involved in epithelial structure homeostasis is yet to be determined.

We performed a comprehensive study of Th22 cells and ILC3 in the sigmoid mucosa of long-term-treated HIV-1–infected individuals with effective control of viremia and immune reconstitution. We found histological evidence of mucosal integrity, despite a significant depletion of IL-22–producing $CD4^+$ T-cells, compared with findings for HIV-1–seronegative subjects. Strikingly, our study revealed preserved numbers of ILCs and normal levels of mucosal IL-22–induced molecules. Thus, our data indicate a dissociation of the mechanisms that maintain Th22 and ILC3 in the human gut, highlighting ILC3 as potentially important therapeutic targets in achieving mucosal integrity in HIV.

METHODS

Studied Cohorts

The study enrolled 37 individuals with a clinical indication to perform colonoscopy (35 of 37 were for colon cancer screening) who were found to be cancer free: 17 were HIV-1–infected individuals who had been receiving ART for a median duration of 8 years with evidence of effective virological and immunological response, and 20 were HIV-1–seronegative patients (Table 1). All individuals gave written informed consent for blood sample and gut biopsy specimen collection and processing. The study was conducted under the approval of the Ethical Boards of the Faculty of Medicine of University of Lisbon, Hospitals Santa Maria and São Bernardo.

Cell Isolation and Culture

Twelve sigmoid biopsy specimens per individual were collected from macroscopically normal mucosa and processed immediately. A total of 7–10 biopsy specimens were digested with collagenase B (10 mg/mL, Roche, Penzberg, Germany) at 37°C and were then mechanically macerated. Lymphocytes were separated by Percoll gradient and cultured for 16 hours at 37°C in complete medium [29]. Cytokine production was assessed after 4-hour stimulation with phorbol myristate acetate plus ionomycin, as previously described [29]. Blood specimens were collected in parallel from 10 HIV-1–infected and 9 control individuals, and peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll-Hypaque centrifugation gradient.

Flow Cytometry

Cells were surface and intracellularly stained (Foxp3 Staining Buffer Set, Ebioscience) as previously described [30], using a panel of antibodies (Supplementary Table 1), and were acquired on Fortessa flow cytometer (BD Biosciences). After exclusion of

Table 1. Characteristics of Subjects in the Human Immunodeficiency Virus Type 1 (HIV-1)–Seronegative Control Cohort and the HIV-1–Infected Cohort

Characteristic	Control (n = 20)	HIV-1 Infected (n = 17)[a]
Male sex	7	13
Age, y	58 (49–63)	58 (52–60)
Length of follow-up, y	NA	12 (8–12)
Length of ART,[b] y	NA	8 (6–11)
Nadir $CD4^+$ T-cell count before ART initiation, cells/μL	NA	339 (162–389)
Viremia, HIV-1 RNA copies/mL, median	NA	<40
$CD4^+$ T-cell count, cells/μL	619 (566–739)	624 (326–774)
$CD8^+$ T-cell count, cells/μL	375 (296–521)	884 (587–1197)[c]
Percentage of naive ($CD45RO^-CCR7^+$) cells[d]		
Among $CD4^+$ T cells	29.4 (23.2–33.4)	31.6 (26.9–43.8)
Among $CD8^+$ T cells	16.1 (11.7–25.3)	15.3 (5.5–26.8)
Percentage of $HLA\text{-}DR^+CD38^+$ cells[d]		
Among memory $CD4^+$ T cells	7.5 (6.3–8.7)	10.8 (7.1–15.4)
Among memory $CD8^+$ T cells	21.1 (17.0–23.6)	29.3 (14.8–46.1)
Percentage of $CD16^+$ cells among $CD14^{bright}$ monocytes[d]	14.4 (12.8–14.7)	16.3 (11.9–28.2)
Percentage of IL-22^+ cells among memory $CD4^+$ T cells[d]	1.5 (0.9–1.5)	1.5 (0.8–1.7)
Percentage of IL-17^+ cells among memory $CD4^+$ T cells[d]	0.7 (0.6–1.5)	0.9 (0.6–1.9)
Percentage of IFN-γ^+ cells among memory $CD4^+$ T cells[d]	10.2 (6.8–38.8)	13.7 (9.2–18.4)

Data are median (interquartile range), unless otherwise indicated. Statistical analysis was performed with the Mann-Whitney *U* test.

Abbreviations: ART, antiretroviral therapy; NA, not applicable.

[a] Subjects were followed at the Departments of Infectious Diseases of the University Hospital de Santa Maria, Lisbon, and Hospital São Bernardo, Setúbal, Portugal.

[b] Triple therapy in all patients (protease inhibitors in 8 of 17).

[c] $P < .05$, compared with controls.

[d] Data are from peripheral blood analysis of 9 control and 10 HIV-1–infected subjects.

dead cells (Live/Dead-LD, Life Technologies, Carlsbad, CA), data analysis was performed with FlowJo (version 9.3.1, Tree Star, Ashland, OR) within gated populations containing ≥150 events (Supplementary Figure 1). Circulating lymphocyte populations were characterized using whole-blood staining and were analyzed for activation markers as previously described [31, 32]. The following antibody combinations were used to identify mucosal ILC and T-cell subsets: IL-17/IL-22/cKIT/IFN-γ/NKp44/LD/CD45/CD3 and IL-17/IL-22/CD8/IFN-γ/CD4/LD/FOXP3/CD3, respectively. Cytokine production by PBMCs was quantified using CCR7/IL-22/IL-17/IFN-γ/CD8/CD45RO/IL-2/CD3 within memory-effector T cells. Unstimulated cells were used to define cytokine cutoffs.

Immunohistochemistry Staining

One sigmoid biopsy specimen and 1 ileum biopsy specimen were embedded in paraffin after preservation in 4% formaldehyde. Three-micrometer sections were stained with antibodies (Supplementary Table 1) after paraffin removal with xylene, rehydration with alcohol, and antigen retrieval by heat. Cells were counted manually in 10 images (original magnification ×400) acquired with a Leica DM2500 bright-field microscope, and the lamina propria area was assessed using ImageJ 1.47i (National Institutes of Health, Bethesda, MD) in blinded samples by 2 researchers. Staining with hematoxylin-eosin, Masson's trichrome, and periodic acid Schiff–Alcian blue (PAS-AB) was concurrently performed, and stained specimens were blindly evaluated by a trained pathologist. Samples were scored according to inflammatory alterations, type of infiltrate, presence or absence of fibrosis, and number of mitotic figures at the crypt base, with scores ranging from 0 to 3.

Immunofluorescence Staining

One sigmoid biopsy specimen and 1 ileum biopsy specimen were frozen in OCT (VWR, Radnor, PA) after 6-hour preservation in 4% paraformaldehyde, followed by 24 hours in 18% sucrose to preserve biopsy structure. Three-micrometer tissue sections were stained by sequential antibody incubations (Supplementary Table 1) after protein blocking. DAPI was used for nuclear counterstaining. Images were acquired with a Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany), using a Plan-Apochromat 20×/0.8 objective; the surface area was measured with ImageJ 1.47i; and positive cells were counted using in-house software, written in MATLAB (Mathworks, Natick, MA), allowing automatic generation of individual report images for cell-counting verification (Figure 1).

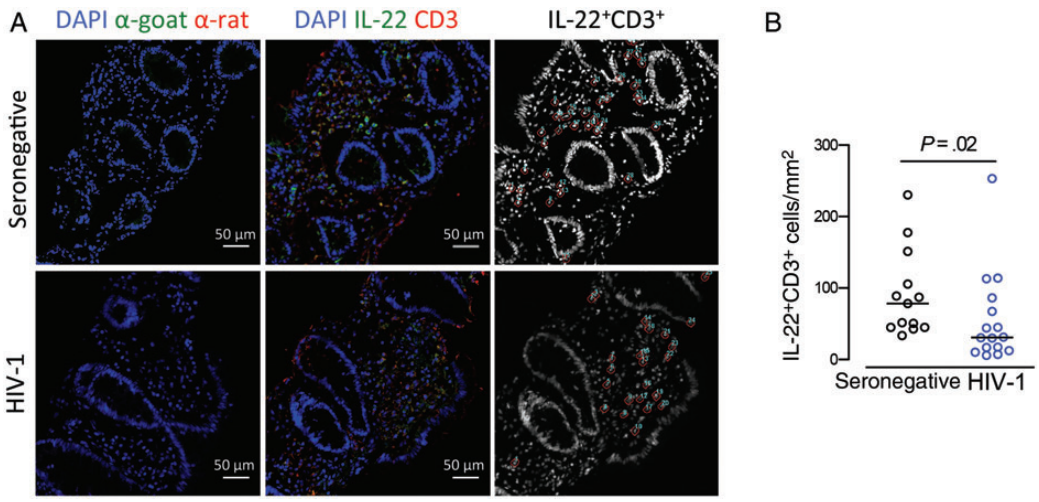

Figure 1. Depletion of interleukin 22 (IL-22)–producing T cells in the sigmoid mucosa of treated human immunodeficiency virus type 1 (HIV-1)–infected individuals. *A*, Representative IL-22 immunostaining in the sigmoid mucosa of HIV-1–infected and control individuals. White bars in figures represent 50 µm. $CD3^+$ cells that express IL-22 were counted using a program developed in-house. Briefly, single-cell nuclei were identified via DAPI by thresholding and particle analysis, and dilated regions of interest (ROIs), based on a user-defined radius, were defined from each nuclei as the corresponding cellular areas for the other 3 channels. For each channel and ROI, a staining was considered positive if a minimum number of pixels (usually 5) were above a given threshold, and a particular combinatorial filter for cell counting was defined on the basis of staining (eg, positive staining in channels A and B but not channel C or positive staining in channels B and C regardless of staining in channel A). Cell numbers are shown in blue. *B*, Number of $IL\text{-}22^+CD3^+$ cells/mm^2 in HIV-1 (n = 16) and seronegative (n = 13) cohorts. Each dot represents 1 individual, and bars represent medians. Groups were compared using Mann-Whitney *U* test. *P* values are shown.

RNA Extraction and Analysis

One sigmoid biopsy specimen and 1 ileum biopsy specimen were stored in RLT buffer (Qiagen, Valencia, CA) immediately after collection. RNA was extracted using the Allprep RNA/DNA mini kit (Qiagen), and 250 ng was used to synthesize complementary DNA (SuperScript III, Life Technologies). Expression levels of *IL22*, *IL17A*, *IL22RA1*, *IL23*, *IL23R*, *IL25*, *TGFB1*, *IL1B*, *IL6*, *MUC1*, *MUC13*, *S100A9*, *REG3G*, *DEFB4A*, *AHR*, and *IDO1* were measured after preamplification with the TaqMan Preamp Master Mix, using TaqMan gene expression assays with an Applied Biosystems 7500 Fast Real-Time PCR System (all from Life Technologies). Results are expressed as ΔCT normalized to the medium CT levels of *GAPDH* and *r18S*. When gene expression was undetectable, a CT value of 40 was used for statistical analysis.

Statistical Analysis

Results are expressed as medians and interquartile ranges (IQRs). Comparisons between 2 groups were made using Mann-Whitney *U* test, using Graph Prism, version 5.0 (GraphPad Software, San Diego, CA). Nonadjusted regression analysis or Spearman correlation was used to identify associations between several parameters, and multivariate linear regression analysis was used to adjust results for age or sex, using Stata, version 12.1 (StataCorp, College Station, TX). Analysis and presentation of distributions of IL-22–producing cells was performed using SPICE, version 5.1 (available at: http://exon.niaid.nih.gov). Comparison of cytokine-producing subset distributions was performed using the Wilcoxon signed rank test and partial permutation test [33]. *P* values of <.05 were considered statistically significant.

RESULTS

IL-22–Producing Cells in the Sigmoid Mucosa

Mucosal IL-22 production was evaluated in sigmoid biopsies from 17 long-term-treated HIV-1–infected individuals with effective suppression of viremia and evidence of immunological reconstitution and 20 HIV-1–seronegative patients (Table 1). The total amount of IL-22 production in the sigmoid mucosa was quantified within hematopoietic cells, defined as $CD45^+$ cells (Supplementary Figure 1).

We observed a significant depletion of IL-22–producing cells in the sigmoid mucosa of treated HIV-1–infected individuals as compared to age-matched controls, in the context of a preserved frequency of $IL\text{-}17^+$ and interferon γ $(IFN\text{-}\gamma)^+$ cells (Supplementary Figure 1). In agreement, the median number of IL-22–positive cells, as determined by immunofluorescence, was significantly reduced in HIV-1–infected individuals as compared to controls (73 cells/mm^2 [IQR, 43–170 cells/mm^2] vs 222 cells/mm^2 [IQR, 152–297 cells/mm^2]; $P = .003$). Although the reduced number of ileum biopsy specimens per subject precluded single-cell isolation, immunohistochemistry studies

revealed similar numbers of IL-22$^+$ cells in the ileum of HIV-1–infected patients when compared to HIV-1–seronegative subjects (Supplementary Figure 2). Our data are in line with previous studies demonstrating a distinct impact of HIV-1 infection of the colon and ileum [34].

Overall, both the frequency and total numbers of IL-22–producing cells were significantly reduced in the sigmoid mucosa in HIV-1 infection even after long-term ART.

Mucosal Th22 Cells

The immunofluorescence staining of IL-22 and CD3 showed a significant depletion of IL-22–producing T cells in HIV-1–positive patients as compared to HIV-1–seronegative subjects (Figure 1).

CD4$^+$ T cells were shown to be the main producers of IL-22 in the human gut [2]. Our analysis of cell suspensions confirmed that adaptive IL-22 production was mostly restricted to mucosal CD4$^+$ T cells, although some subjects (5 of 15 HIV-1–infected individuals and 4 of 13 controls) featured a few IL-22–producing CD8$^+$ T cells (<2.5% of the CD8$^+$ T-cell population; Supplementary Figure 1).

We found that the frequency of IL-22–producing cells within the CD4$^+$ T-cell subset was significantly decreased in treated HIV-1–positive individuals (Figure 2*A* and 2*B*), even after adjustment for the total number of mucosal CD4$^+$ T cells ($R = -5.9$; $P = .008$).

Of note, our long-term-treated HIV-1–infected cohort showed no significant reduction of CD4$^+$ T-cell counts in the

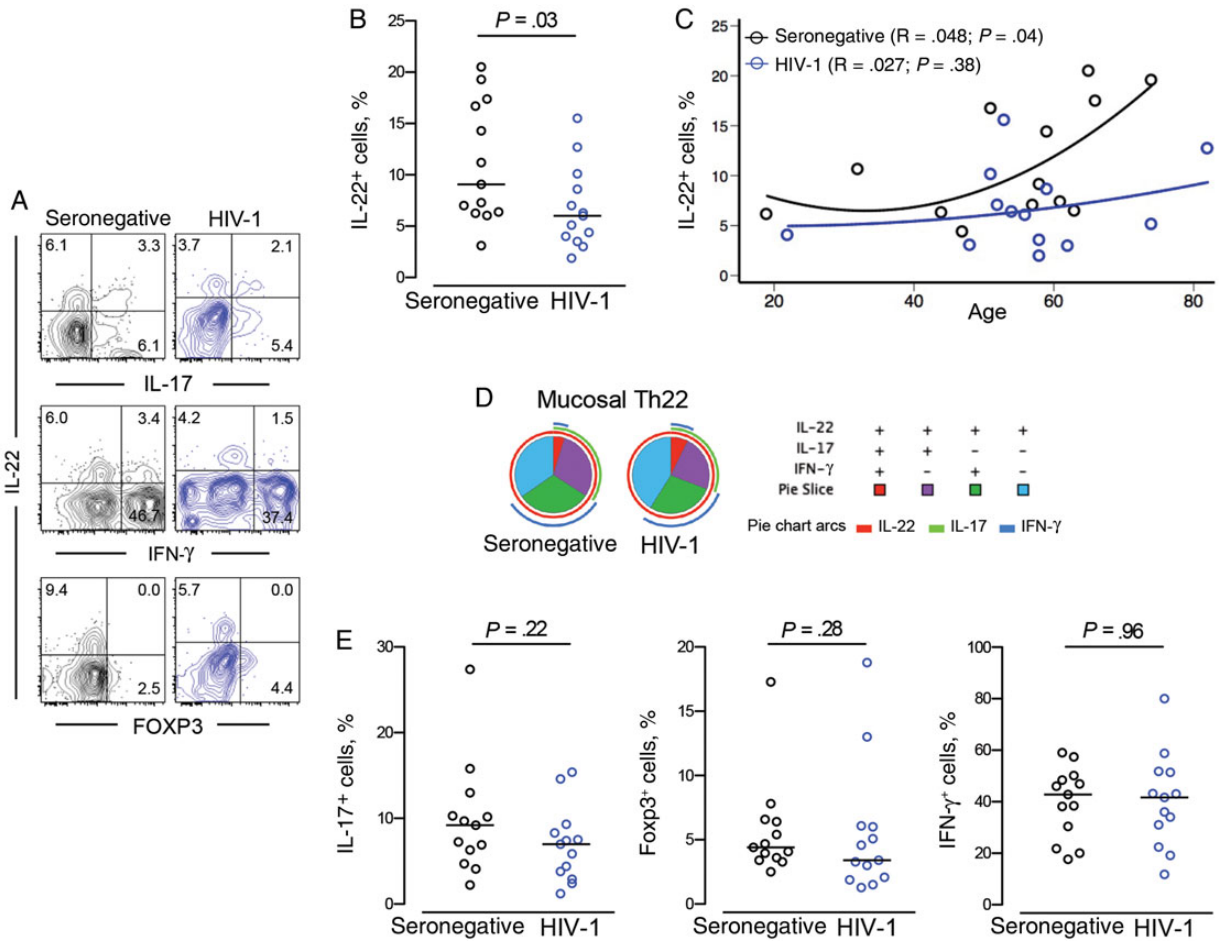

Figure 2. Interleukin 22 (IL-22)–producing CD4$^+$ T cells in the sigmoid mucosa. *A*, Representative dot plots of IL-22, interferon γ (IFN-γ), interleukin 17 (IL-17), and FOXP3 expression within gated mucosal CD4$^+$ T cells from human immunodeficiency virus type 1 (HIV-1)–infected and control individuals. Cytokine production was assessed upon phorbol myristate acetate–ionomycin stimulation of cells isolated from biopsy specimens of macroscopically normal mucosa. Numbers inside quadrants represent the frequency of positive cells. *B*, Frequency of IL-22$^+$ cells among CD4$^+$ T cells from the HIV-1–positive (n = 13) and seronegative (n = 13) cohorts and its association with age. *C*, Results of multiple linear regression analysis used to calculate *R* coefficients, including a quadratic term for age. *D*, Distribution of IL-17 and IFN-γ production among total IL-22–producing CD4$^+$ T cells in HIV-1–positive and seronegative cohorts, determined using SPICE software 5.0. *E*, IL-17$^+$, FOXP3$^+$, and IFN-γ$^+$ cells among CD4$^+$ T cells within the HIV-1–positive and control cohorts. Each dot represents an individual, and bars represent median. Groups were compared using the Mann-Whitney *U* test, and *P* values are shown. Th22, T-helper type 22 cells.

sigmoid mucosa, though an expansion of $CD8^+$ T cells was still observed (Supplementary Figure 1).

The levels of mucosal Th22 cells were not related to peripheral blood $CD4^+$ T-cell counts ($R = -0.031$; $P = .89$; n = 22) or to the frequency of circulating IL-22–producing $CD4^+$ T cells ($R = 0.1$; $P = .72$; n = 17), with the latter values being similar in the HIV-1–infected and control cohorts (Table 1). Importantly, there was no association between mucosal Th22 frequency and nadir $CD4^+$ T-cell count before ART or by length of therapy.

Interestingly, aging was associated with a progressive increase in IL-22–producing cells within mucosal $CD4^+$ T cells in healthy subjects (Figure 2*C*). This relationship was not observed in the HIV-1–infected cohort (Figure 2*C*), supporting a disturbed homeostasis of this subset in the sigmoid mucosa, even after long-term receipt of ART.

Nevertheless, the proportion of Th22 cells that concomitantly produce IL-17 and/or IFN-γ revealed no significant imbalances in HIV-1–infected patients as compared to healthy individuals (Figure 2*D*), with all IL-22–producing $CD4^+$ T-cell subsets underrepresented in the HIV-1–infected cohort (Supplementary Figure 1).

Mucosal Th22 cells had significantly more coexpression of IL-17 and/or IFN-γ than the circulating counterparts in both cohorts (data not shown). Thus, despite the major Th22 cell depletion found in HIV-1–infected patients, the factors governing the balance of these Th22 cell subpopulations seemed to be operating as in controls.

The frequency of the other main $CD4^+$ T-cell subsets, namely $IL\text{-}17^+$, $IFN\text{-}\gamma^+$, and $FOXP3^+$, were similar in the 2 cohorts (Figure 2*E*), supporting a preferential depletion of Th22 cells. Accordingly, immunohistochemistry analysis revealed similar median numbers of $IL\text{-}17^+$ cells in HIV–infected and HIV-1–seronegative cohorts (123 cells/mm^2 [IQR, 60–202 cells/mm^2; n = 15) vs 82 cells/mm^2 [IQR, 77–149 cells/mm^2; n = 15]; $P = .74$) and $FOXP3^+$ cells (37 cells/mm^2 [IQR, 26–87 cells/mm^2; n = 17] vs 54 cells/mm^2 [IQR, 14–87 cells/mm^2; n = 17]; $P = .89$).

In conclusion, long-term-treated HIV-1–infected individuals exhibited a significant depletion of Th22 cells in the sigmoid mucosa.

Levels of Molecules Known to Regulate IL-22 Production in the Sigmoid Mucosa

Next, we investigated possible alterations in the expression levels of molecules known to modulate IL-22 production in the gut. We found no alterations in the mRNA expression levels of *IL23*, *IL23R*, *IL6*, *IL1B*, *AHR*, *IL25*, *IDO1*, and *TGFB1* (Figure 3). Moreover, the levels of these transcripts were not significantly associated with the frequency of IL-22–producing cells, either within total hematopoietic cells or $CD4^+$ T cells ($P > .3$). Similar findings were obtained in ileum biopsies (Supplementary Figure 2).

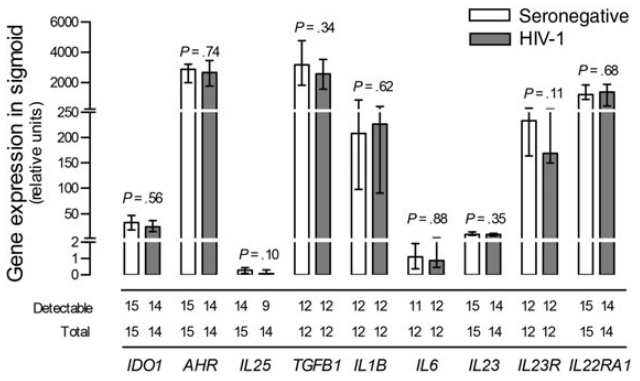

Figure 3. Levels of expression of genes known to modulate interleukin 22 (IL-22) production and/or function. *IL23, IL25, AHR, IDO1, TGFB1, IL6, IL1B, IL23R,* and *IL22RA1* messenger RNA levels in total RNA extracted from sigmoid biopsy specimens of human immunodeficiency virus type 1 (HIV-1)–infected and seronegative individuals. Numbers below indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*. Bars represent medians and interquartile ranges. Comparisons were made using the Mann-Whitney *U* test. *P* values are shown, supporting the lack of any significant differences.

Thus, the expression levels of the main regulators of mucosal IL-22 production were preserved in treated HIV-1–positive individuals, suggesting they feature a normal IL-22–permissive environment.

IL-22–Producing Non-T Cells and ILC3 in the Sigmoid Mucosa

Next we investigated IL-22 production by non-T cells, and found that it was comparable in the 2 cohorts (Figure 4*A*). Notably, HIV-1–infected and control individuals featured a similar profile of concomitant production of IL-17 and/or IFN-γ by non-T cells, with a marked prevalence of cells producing only IL-22 in both cohorts.

Additionally, we performed a triple immunofluorescence staining for CD3, RORγt, and IL-22 in the sigmoid mucosa and confirmed that the numbers of $CD3^-ROR\gamma t^+$ cells and $CD3^-ROR\gamma t^+IL\text{-}22^+$ cells were similar in the 2 cohorts (Figure 4*B*).

The main source of innate IL-22 in the gut is a subset of ILCs that express the natural cytotoxic receptor NKp44: ILC3. HIV-1–infected individuals showed a preserved frequency of mucosal ILC3 cells, defined by the expression of cKIT and NKp44 within $CD3^-$ cells, as compared to HIV-1–seronegative patients (Figure 5*A*). These cells expressed RORγt (Figure 5*B*), which is essential for their development and function [35]. Moreover, similar to healthy subjects, the mucosal ILCs of HIV-1–positive individuals also expressed high levels of the IL-7 and interleukin 2 receptor α chains (CD127 and CD25, respectively; Supplementary Figure 1) [36]. In agreement with previous data [37], we found that the ILC3 subset was the main producer of

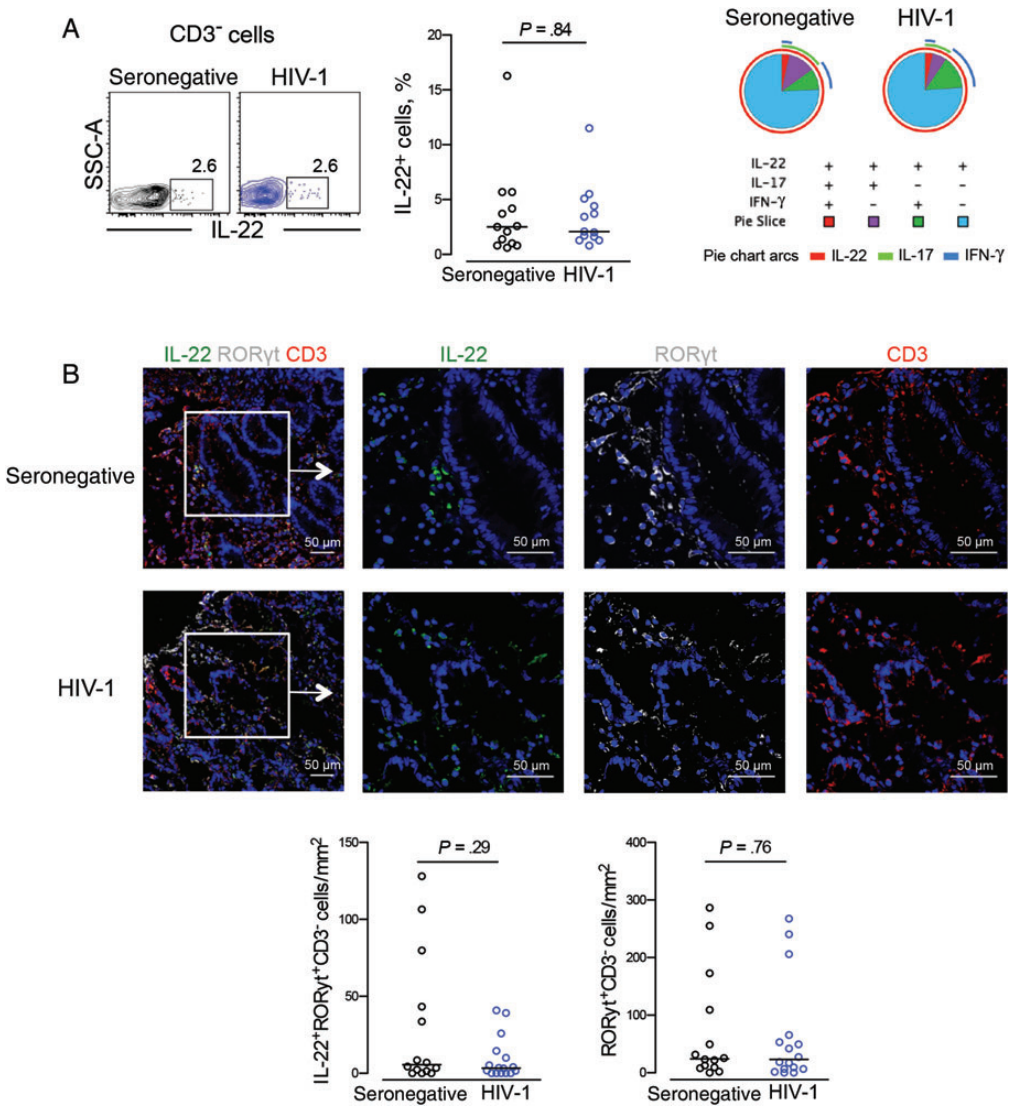

Figure 4. Interleukin 22 (IL-22) production by non-T cells in sigmoid mucosa. *A*, Flow cytometry analysis of IL-22 production within gated $CD3^-$ cells from HIV-1–infected and control subjects. Representative dot plots show the proportion of positive cells, depicted inside boxes (left); frequency within human immunodeficiency virus type 1 (HIV-1)–infected (n = 13) and control (n = 13) cohorts (center); and the distribution of interleukin 17 (IL-17) and interferon γ (IFN-γ) production among these cells in both cohorts, using SPICE software 5.0 (right). *B*, Immunofluorescence quantification of IL-22–producing cells in sigmoid biopsy specimens from HIV-1–infected and control individuals, showing representative images of IL-22, RORγt, and CD3 staining, with white bars indicating 50 μm (top), and mucosal counts of $ROR\gamma t^+CD3^-$ and $IL\text{-}22^+ROR\gamma t^+CD3^-$ cells in HIV-1–infected (n = 16) and control (n = 14) cohorts (bottom). Each dot represents an individual, and bars indicate medians in the graphs. Analysis was performed using the Mann-Whitney *U* test, and *P* values are shown.

IL-22 in both healthy and HIV-1–infected individuals, with no skewing toward IFN-γ production (Figure 5*B*).

Of note, a multivariate regression analysis including HIV status and sex as cofactors confirmed that sex did not bias our results regarding the frequencies of ILC ($P = .42$), IL-22–producing non-T cells ($P = .49$), or $CD4^+$ T cells ($P = .18$).

Hence, in contrast to Th22 cell depletion, treated HIV-1–infected patients featured a preserved frequency of innate IL-22–producing cells.

Epithelial Preservation in Long-Term-Treated HIV-1–Positive Individuals

Finally, we investigated the degree of sigmoid epithelial disruption in the context of marked Th22 cell depletion but preserved ILC3.

Initially, we quantified expression of IL-22–target genes, which are likely to help maintain epithelial integrity. We found that the genes for mucins (*MUC1* and *MUC13*) and antimicrobial peptides (*REG3G*, *S100A9*, and *DEFB4A*)

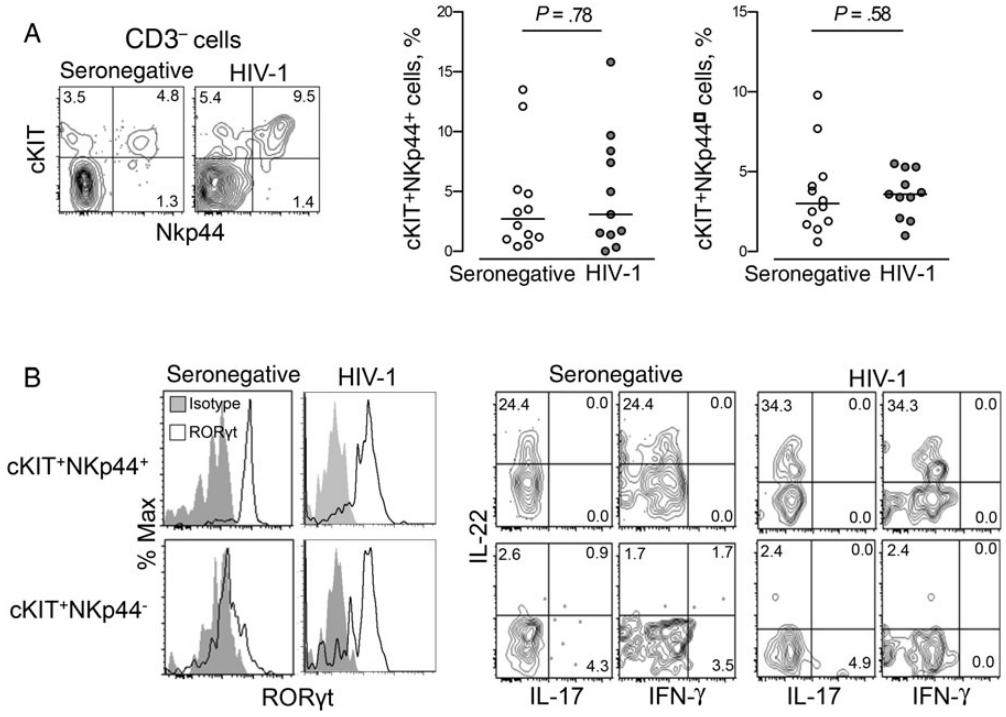

Figure 5. Innate lymphoid cells in the sigmoid mucosa. *A*, Representative dot plots of cKIT and NKp44 expression within gated $CD3^-$ cells from human immunodeficiency virus type 1 (HIV-1)–infected and control individuals. Numbers inside quadrants represent frequency of positive cells. Graphs show the frequency of $cKIT^+NKp44^-$ and $cKIT^+NKp44^+$ cells among $CD3^-$ cells from HIV-1–infected (n = 11) and control (n = 12) cohorts. Each dot represents an individual, and bars indicate medians in the graphs. Analysis was performed using the Mann-Whitney *U* test; *P* values are shown. *B*, Representative analysis of RORγt expression, as well as interleukin 22 (IL-22), interleukin 17 (IL-17), and interferon γ (IFN-γ) production within $cKIT^+NKp44^-$ and $cKIT^+NKp44^+$ gated $CD3^-$ cells from the sigmoid mucosa of HIV-1–infected and control subjects.

were similarly expressed in the HIV-1–infected and control cohorts (Figure 6*A*). The strong association we observed between the levels of transcripts for *IL22RA1* and *MUC13* (Figure 6*B*) is worth emphasizing, as it supports the role of IL-22 in the induction of this particular molecule. Ileum biopsy specimens from both cohorts also featured similar levels of these transcripts (Supplementary Figure 2). Furthermore, there was no evidence of compromised tight junction integrity, as assessed by levels of expression of zonula occludens 1 (ZO-1; Figure 6*C*).

Additionally, blinded examination of specimens stained with hematoxylin-eosin, Masson's trichrome, and PAS-AB revealed no significant differences between the 2 cohorts with respect to crypt morphology, number and type of cell infiltrates, and number of epithelial mitotic events (Figure 6*D*). Mucin production was apparently preserved in all samples, and significant collagen deposition was only found in biopsy specimens from 2 HIV-1–infected individuals (data not shown).

Interestingly, our long-term-treated HIV-1–infected cohort showed no significant increase in the expression of activation markers in circulating $CD4^+$ and $CD8^+$ T-cell subsets or an expansion of $CD16^+$ cells within $CD14^{bright}$ monocytes (Table 1). The lack of upregulation of these markers, which have been associated with microbial translocation, was in agreement with preserved epithelial integrity, as suggested by our histological data and the levels of antimicrobial peptides.

Altogether, our study of long-term-treated HIV-1 infection provides evidence for full epithelium integrity in the context of significant Th22 cell depletion and preserved ILC3.

DISCUSSION

Maintenance of intestinal homeostasis is linked to IL-22 production in the lamina propria [26]. This study provided evidence favoring distinct regulation of the 2 main IL-22 producers in the human gut, $CD4^+$ T cells (Th22 cells) and ILC3. We showed that IL-22–mediated processes were functioning in long-term-treated HIV-1–infected patients, despite evidence of Th22 cell depletion. Conversely, innate IL-22 production was unaltered, suggesting a role for ILC3 in ensuring epithelial integrity.

Gut disruption is a hallmark of HIV-1 infection, and the restoration of intestinal integrity upon ART initiation is still controversial [18]. Using a comprehensive approach combining tissue structure analyses, cell counts, and relative proportion of functionally characterized populations, as well as quantification

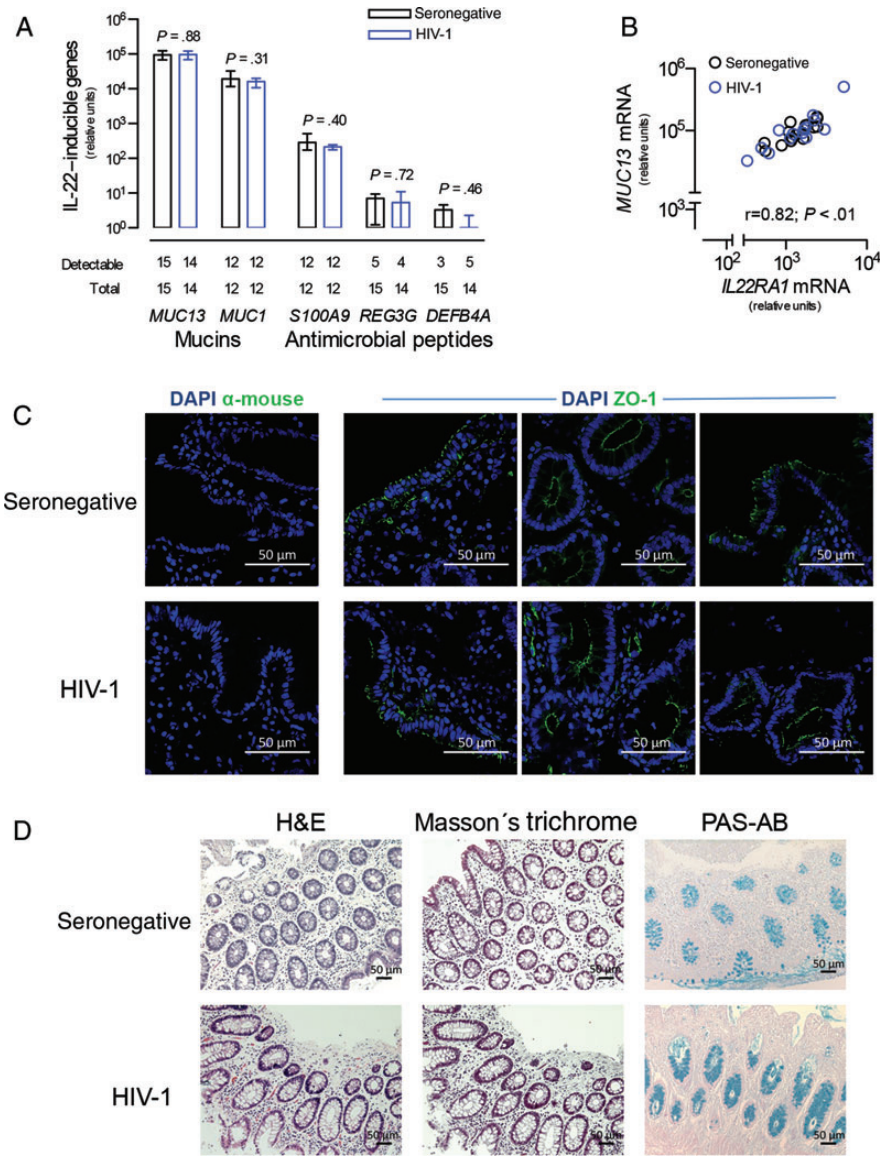

Figure 6. Levels of expression of interleukin 22 (IL-22)–induced genes and sigmoid mucosa integrity. *A*, Messenger RNA (mRNA) levels of mucins and antimicrobial molecules induced by IL-22 in biopsy specimens from human immunodeficiency virus type 1 (HIV-1)–infected and control cohorts. Numbers indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. Results are expressed in relative units, normalized to the mean CT levels of *GAPDH* and *r18S*, with bars representing medians and interquartile range. Comparisons were made using the Mann-Whitney *U* test, and *P* values are shown. *B*, Association between levels of expression of *IL22RA1* and *MUC13* mRNA. Spearman correlation was used to calculate the r coefficient and *P* values. *C*, Illustrative immunofluorescence staining of zonula occludens 1 (ZO-1) in 3 different HIV-1–infected and seronegative individuals. Findings were used to quantify the median percentage of the epithelial area staining positive for ZO-1 in HIV-1–infection (21% [interquartile range, 16%–30%]; n = 7) and control (22% [interquartile range, 20%–28%]; n = 5) groups. *D*, Representative hematoxylin-eosin (H-E), Masson's trichrome, and periodic acid Schiff–Alcian blue (PAS-AB) staining of sigmoid biopsy specimens from HIV-1–infected and control individuals. Findings were used to score the histological alterations. The mean value was 1.1 for HIV-1–positive subjects (n = 13) and 1.4 for HIV-1–seronegative patients (n = 15). Bars = 50 µm.

of expression of key genes implicated in gut homeostasis, we demonstrated the absence of major GALT disturbances in patients starting ART even in advanced stages of immunodeficiency. In summary, we documented: (1) an absence of $CD4^+$ T-cell depletion, reaching normal absolute counts in the lamina propria despite the persistence of increased $CD8^+$ T-cell numbers; (2) normal numbers, as well as balance, of regulatory T cells and $CD4^+$ T cells that produce the proinflammatory cytokines IL-17 and/or IFN-γ; (3) evidence of control of local inflammation provided by lack of overexpression of relevant genes (eg, *IL6*, *IL1B*, *TGFB1*, and *IDO1*); (4) preserved epithelial structure in the sigmoid and ileum; and (5) absence of systemic

markers associated with microbial translocation, in our long-term-treated HIV-1–infected cohort.

Importantly, our study revealed the maintenance of several processes that are known to rely on IL-22, namely ZO-1–associated tight junction integrity and production of regulins, S100, defensins, and mucins, all of which are essential for preserving intestinal homeostasis [38]. This central role of IL-22 in tissue repair is in line with several reports in other clinical contexts and models of disease [26].

Strikingly, we documented mucosal integrity despite the significant Th22 cell depletion in the sigmoid mucosa of long-term-treated HIV-1–infected individuals. To our knowledge, there is only 1 report that evaluated IL-22 production in the human sigmoid mucosa [27]. This cross-sectional study described Th22 cell depletion associated with disruption of epithelial integrity that was apparently recovered in treated patients. However, this recovery should be cautiously interpreted because only 8, much younger controls were evaluated. This is particularly critical given our finding of a positive correlation between the frequency of Th22 cells among $CD4^+$ T cells and age in HIV-1–seronegative subjects. Thus, the use of a large cohort of age-matched controls strengthens our observation of Th22 cell depletion after long-term ART in HIV-1 infection.

This preferential Th22 cell depletion could not be linked to an altered expression of factors that distinctly govern IL-22 and IL-17 production, namely AHR [24] and TGF-β [25]. The possibility of distinct homing requirements should be explored in future studies.

Interestingly, irrespective of HIV-1 infection status of the subjects, our parallel study of circulating and mucosal Th22 cells revealed higher coproduction of IL-17 and/or IFN-γ in the latter group. It is plausible that cytokine patterns are influenced by the mucosal environment, in which case the similarity of the Th22 profile in the 2 cohorts represents an additional argument in favor of gut integrity in long-term-treated HIV-1–infected patients. In agreement, no skewing toward IFN-γ production was found in ILC3 of the HIV-1 cohort, in contrast with published data on untreated SIV infection in nonhuman primates [39].

Importantly, ILC3 were not depleted in the treated HIV-1 cohort, supporting a role for distinct factors governing the homeostasis of the 2 main IL-22–producing subsets in the gut mucosa. Given the lower frequency of these cells and the limited amount of tissue obtained during human studies, data on the relative contribution of ILC3 and Th22 cells to IL-22 production are still scarce. In contrast, murine models of colitis have generated data in favor of distinct regulation of these 2 subsets during disease [40]. Moreover, ILCs were shown to maintain epithelial preservation in the absence of IL-22 production by T cells [40]. Thus, it is plausible that the relative proximity to epithelial cells, rather than the actual frequency in lamina propria, may determine the role played by ILCs in maintaining epithelial integrity and may also help preserve epithelial stem cells [3]. Nevertheless, although we were able to identify ILC3 in the human gut, the structure of the frozen tissue biopsy specimens was insufficiently preserved to allow us to assess their relative localization in relation to Th22 cells. Notably, in addition to IL-22 production, ILC3 were recently reported to control mucosal inflammation by modulating local $CD4^+$ T-cell activation [15]. Thus, ILC3 may represent an important population to target in the context of HIV-1 infection, since a hyperactivated state is believed to underlie mucosal damage.

Overall, mucosal homeostasis, including IL-22–mediated mechanisms essential for epithelial integrity, could mainly rely on ILCs, as suggested by our data on long-term-treated HIV-1–infected patients. Thus, the therapeutic targeting of ILCs may represent an important strategy to promote intestinal recovery.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the individuals who participated in the study; the physicians, for their follow-up and collection of clinical data, particularly Joana Fernandes, Manuela Doroana, Sérgio E. Paulo, Nuno Janeiro, and Francisco Antunes from the Department of Infectious Diseases of Hospital de Santa Maria, Lisbon; the collaboration of the Gastroenterology Departments of Hospital de Santa Maria, Lisbon, and Hospital de S. Bernardo, Setúbal, for the endoscopic studies; Paula Matoso and Vânia Silva, for the technical assistance in molecular biology; and Bruno Silva-Santos, Helena Nunes-Cabaço, Iris Caramalho, and Rui M. M. Victorino, for the critical discussion of the manuscript.

Financial support. This work was supported by the Fundação para a Ciência e Tecnologia (FCT) and the Programa Operacional Ciência e Inovação 2010 (PIC/IC/82712/2007 to A. E. S). Scholarships were provided to S. M. F., A. R. P., and R. B. F. by the FCT.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Supplementary Table 1. Antibodies used

Monoclonal Antibodies used in Flow Cytometry Staining

Antibody	Fluorochrome	Clone	Source
IL-17	FITC	eBIO54DEC17	eBioscience[a]
IL-22	PE	22URTI	eBioscience
FOXP3	V450	PCH101	eBioscience
CD117 (cKIT)	PerCP	104D2	eBioscience
CD45	V450	2D1	eBioscience
CD45RO	APC-Cy7	UCHL1	eBioscience
CD3	V500	UCHT1	eBioscience
CD4	APC	RPA-T4	eBioscience
CD8	PerCP	RPA-T8	eBioscience
CD14	PE-Cy7	61D3	eBioscience
CD19	APC-efluor780	HIB19	eBioscience
CD16	FITC	eBioCB16	eBioscience
CD38	PE	HB7	eBioscience
HLA-DR	PerCP-efluor 710	L243	eBioscience
IFNy	PE-Cy7	4S.B3	Biolegend[b]
CD123	V450	6H6	Biolegend
Nkp44	APC	243415	R&D[c]
CCR7	FITC	150503	R&D
CD25	PE-Cy7	M-A251	BD Bioscience[d]
CD45RO	APC-H7	UCHL1	BD Bioscience
CD56	PE	My31	BD Bioscience
CD11c	APC	B-Ly6	BD Bioscience
CD3	V450	UCHT1	BD Bioscience
CD4	V500	RPA-T4	BD Bioscience

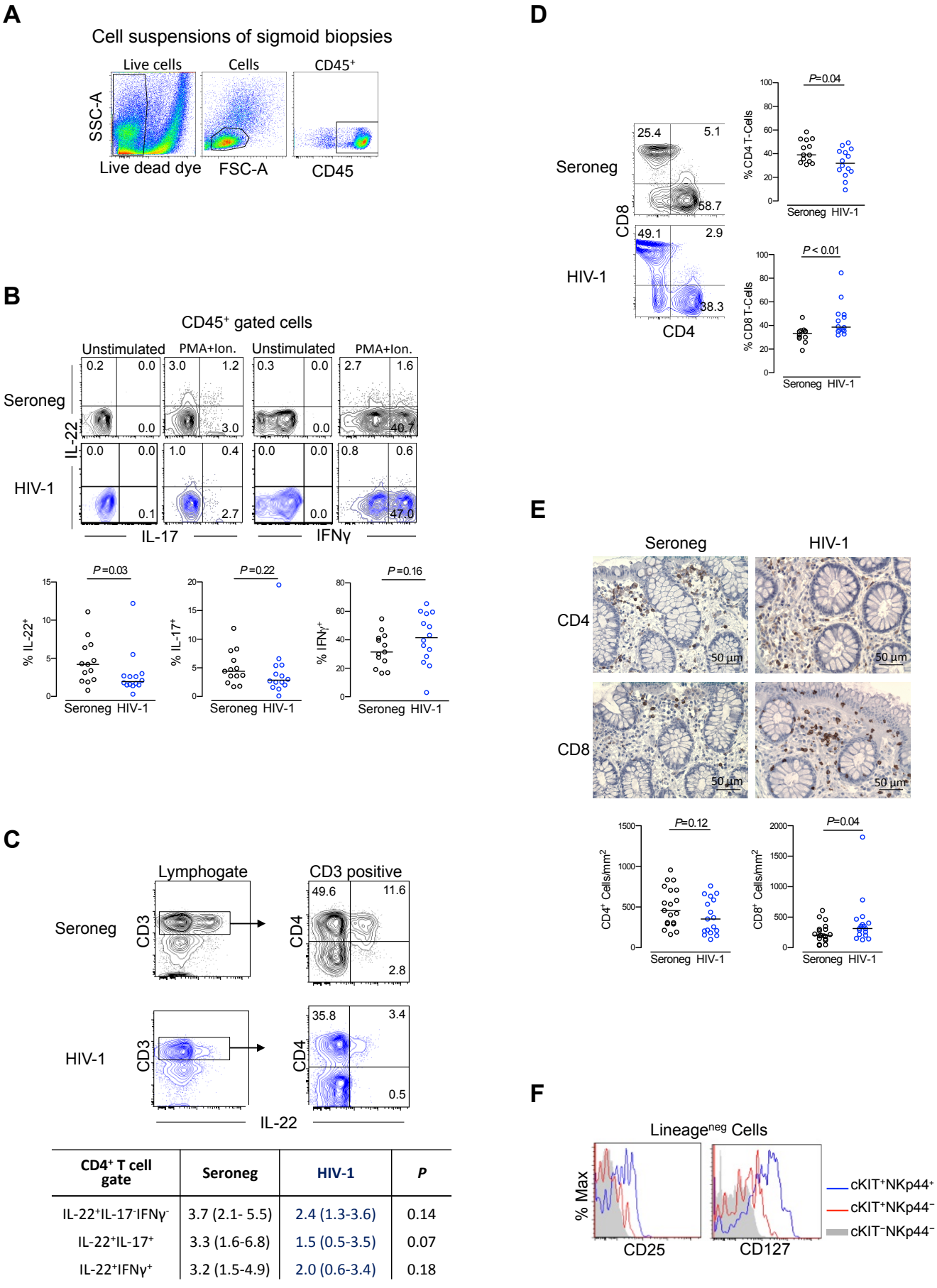
Antibodies used in Immunofluorescence Staining

	Antibody	Source
Primary antibodies	Rabbit anti-RORγt	Abcam[e]
	Goat anti-IL-22	Santa Cruz Biotechnology[f]
	Rat anti-CD3	Acris[g]
	Mouse anti-ZO-1	BD Biosciences
	Alexa Fluor 488 Anti-Goat	Molecular Probes[h]
Secondary antibodies	Alexa Fluor 488 Anti-Mouse	Molecular Probes
	Alexa Fluor 546 Anti-Rabbit	Molecular Probes
	Alexa Fluor 647 Anti-Rat	Molecular Probes

Antibodies used in Immunochemistry Staining

Antibody	Clone	Source
CD4	4B12	Leica Biosystems[i]
CD8	1A5	Leica Biosystems
FOXP3	236/E7	Leica Biosystems
IL17	polyclonal	Santa Cruz Biotechnology

[a]eBioscience, San Diego, CA; [b]Biolegend, San Diego, CA; [c]R&D, Minnneapolis, MN, USA; [d]BD Bioscience, San Jose, CA; [e]Abcam, Cambridge, UK; [f]Santa Cruz Biotechnology, Dallas, USA; [g]Acris, Herford, Germany; [h]Molecular Probes, Life Technologies, Carlsbad, USA; [i]Leica Biosystems, Wetzlar, Germany.



CD4+ T cell gate	Seroneg	HIV-1	P
IL-22+IL-17-IFNγ-	3.7 (2.1- 5.5)	2.4 (1.3-3.6)	0.14
IL-22+IL-17+	3.3 (1.6-6.8)	1.5 (0.5-3.5)	0.07
IL-22+IFNγ+	3.2 (1.5-4.9)	2.0 (0.6-3.4)	0.18

Supplementary Figure 1*. Analysis of sigmoid mucosa*

A) Flow cytometry analysis of mucosal cells illustrating the sequential gating strategy of cell suspensions obtained from sigmoid biopsies of macroscopically normal mucosa showing dot-plots of dead cell exclusion, lymphogate definition accordingly to SSC-A and FSC-A, and gating on CD45+ cells.

B) Representative dot-plots of IL-22, IL-17 and IFN-γ staining within live $CD45^+$ population, with and without PMA-ionomycin stimulation, in a seronegative and an HIV-1-infected individual, with graphs showing the frequency of cytokine producing cells in HIV-1-infected (n=14) and seronegative (n=13) cohorts.

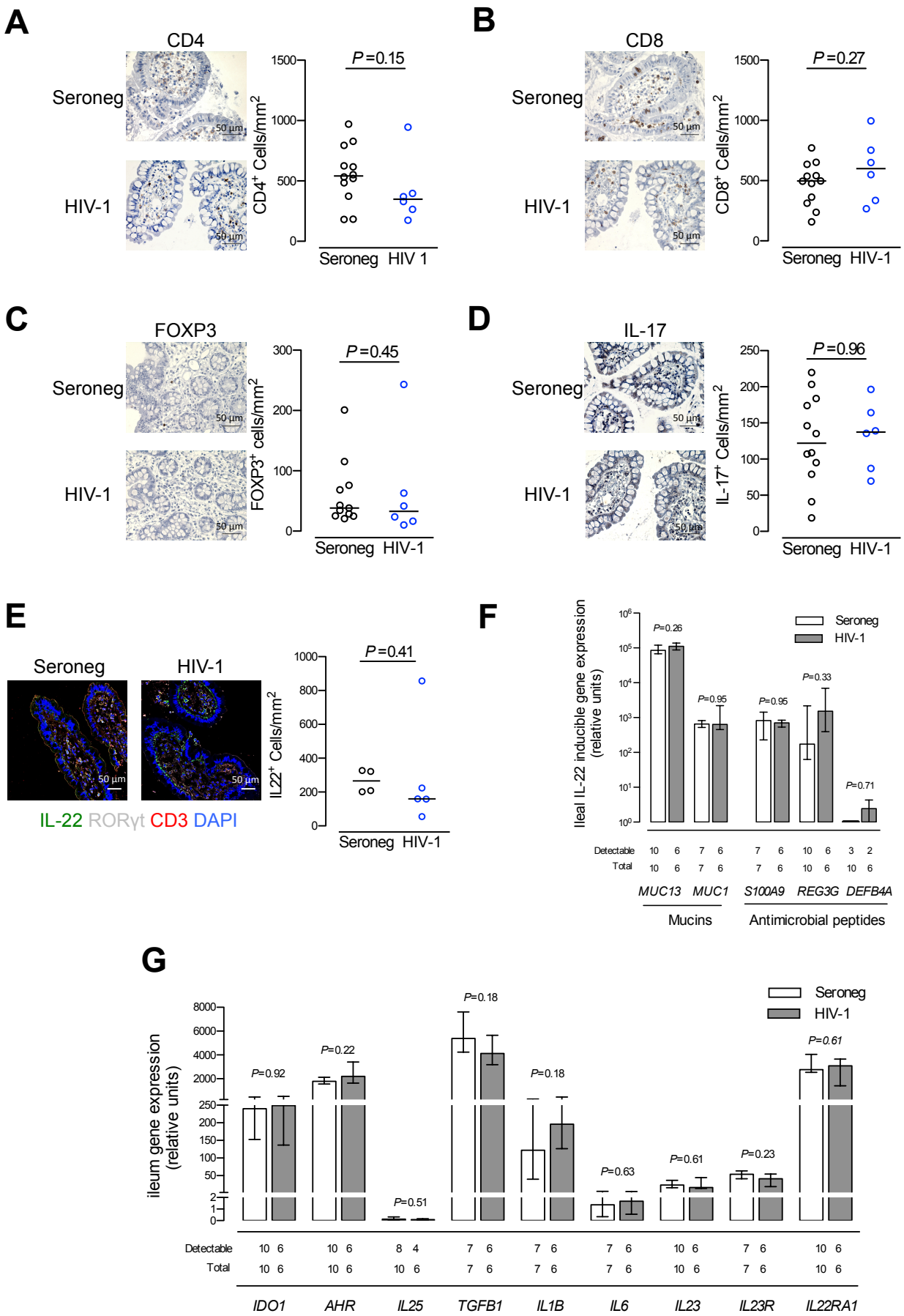
C) Illustrative dot-plots of IL-22 and CD3 staining within lymphogate, as well as of IL-22 and CD4 staining within total T-cells in a seronegative and an HIV-1-infected subject; the frequency of the IL-22-producing subpopulations within total CD4 T-cells in HIV-1-infected (n=13) and seronegative (n=13) cohort is tabulated bellow.

D) Representative dot-plots of CD4 and CD8 expression analysed within total $CD3^+$ cells in a seronegative and an HIV-1-infected subject; graphs show the frequency of these subsets in seronegative (n=13) and HIV-1-infected (n=14) cohorts.

E) Representative immunohistochemistry images of CD4 and CD8 staining (black bars indicate 50μm) in a seronegative and an HIV-1-infected subject; graphs show the corresponding counts of CD4 and CD8 positive cells in seronegative (n=19) and HIV-1 (n=17) cohorts.

F) Expression of the α-chains of the IL-7 receptor (CD127) and the IL-2 receptor (CD25) by innate lymphoid cells isolated from the sigmoid mucosa of an HIV-1-infected individual; analysis was performed after gating in lineage (CD3, CD19, CD14, CD16, CD123) negative cells, and histograms compare the expression of CD127 and CD25 within $cKIT^+NKp44^+$, $cKIT^+NKp44^-$ and $cKIT^-NKp44^-$ cells.

In the dot-plots, numbers inside quadrants represent the frequency of positive cells. In the graphs, each dot represents an individual, and bars indicate medians. Analysis was performed using Mann-Whitney U test and all *P* values are shown.


A
CD4
Seroneg
HIV-1
50 µm
CD4+ Cells/mm2
1500
1000
500
0
P=0.15
Seroneg HIV 1
B
CD8
Seroneg
HIV-1
50 µm
CD8+ Cells/mm2
1500
1000
500
0
P=0.27
Seroneg HIV-1
C
FOXP3
Seroneg
HIV-1
50 µm
FOXP3+ cells/mm2
300
200
100
0
P=0.45
Seroneg HIV-1
D
IL-17
Seroneg
HIV-1
50 µm
IL-17+ Cells/mm2
250
200
150
100
50
0
P=0.96
Seroneg HIV-1
E
Seroneg
HIV-1
50 µm
IL-22 RORγt CD3 DAPI
IL22+ Cells/mm2
1000
800
600
400
200
0
P=0.41
Seroneg HIV-1
F
Ileal IL-22 inducible gene expression (relative units)
Seroneg
HIV-1
P=0.26
P=0.95
P=0.95
P=0.33
P=0.71
Detectable 10 6 7 6 7 6 10 6 3 2
Total 10 6 7 6 7 6 10 6 10 6
MUC13 MUC1 S100A9 REG3G DEFB4A
Mucins
Antimicrobial peptides
G
ileum gene expression (relative units)
Seroneg
HIV-1
P=0.92
P=0.22
P=0.51
P=0.18
P=0.18
P=0.63
P=0.61
P=0.23
P=0.61
Detectable 10 6 10 6 8 4 7 6 7 6 7 6 10 6 7 6 10 6
Total 10 6 10 6 10 6 7 6 7 6 7 6 10 6 7 6 10 6
IDO1 AHR IL25 TGFB1 IL1B IL6 IL23 IL23R IL22RA1

Supplementary Figure 2. ***Analysis of ileum mucosa.***

Ileum biopsies obtained from 6 HIV-1-infected and 12 seronegative individuals, comparable in terms of age (54 years [52-60] versus 55 years [49-60], respectively) and CD4 counts (580 cells/μL [411-630] versus 672 cells/μL [584-822], respectively), with an over-representation of males in the HIV-1 group (5/6 versus 5/12). Representative images of CD4 (A), CD8 (B), FOXP3 (C) and IL-17 (D), and related cell counts in the ileum mucosa of HIV-1-infected (n=6) and control individuals (n=12) are shown. E) Illustrative immunofluorescence staining of IL-22, RORγt and CD3 in the ileum mucosa of HIV-1 and seronegative subjects. Total number of IL-22^{+} cells/mm^2 per group are shown. Ileum mRNA levels of genes induced by IL-22 (F), and of genes known to modulate IL-22 production and/or function (G), with numbers of samples evaluated and of samples with detectable levels of the respective genes depicted below. Results are expressed in relative units, normalized to the mean CT levels of GAPDH and r18S. Each dot represents an individual and bars represent medians in the graphs. Bar graphs represent medians and interquartile range. Comparisons were made using Mann-Whitney U test and *P* values are shown.

program, contract LSHP-CT-2006-037570, BMS, MSD and Janssen Pharmaceuticals N.V.

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Received: 18 September 2012; revised: 25 September 2013; accepted: 25 September 2013.

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DOI:10.1097/QAD.0000000000000100

Gut disruption in HIV-2 infection despite reduced viremia

HIV-2 infection is highly prevalent in West Africa and has been increasingly observed in non-African countries, mostly associated with migratory populations [1]. It has a much more benign course and lower viremia than HIV-1 [2], though with similar clinical spectra. Half of the HIV-2 infected patients with less than 200 $CD4^+$ T-cells/μl exhibit undetectable viremia, despite harbouring numbers of infected cells comparable to their HIV-1 counterparts [3]. Moreover, $CD4^+$ T-cell loss occurs in direct association with progressive immune activation in both infections, though the depletion rate is much slower in HIV-2 [2,4]. HIV-1 disease progression has been linked to disruption of gut-associated lymphoid tissue (GALT) and increased levels of microbial translocation, leading to systemic immune activation. There are currently no data on the impact of HIV-2 on GALT. Here, we provide evidence of HIV-2 replication in the gut despite the low viremia, which was associated with major mucosal disruption and $CD4^+$ T-cell depletion that recovered upon antiretroviral treatment (ART).

An 18-year-old man, from Guinea-Bissau, with HIV-2 infection possibly acquired by vertical transmission, presented with mild diarrhoea since the first year of life. Colonoscopy showed loss of haustra and rectal ulcers, with moderate lymphoplasmocytic and neutrophilic infiltrates in rectal biopsies (Fig. 1a,b). Circulating $CD4^+$ T-cells were low (19%, 103 cells/μl), in association with hyperimmune activation (Fig. 1c) and detectable viremia (4575 RNAcopies/ml), albeit at levels much lower than those found in HIV-1 infection. There was a significant depletion of $CD4^+$ T-cells and an increase in CD8 T-cells in sigmoid lamina propria (Fig. 1a). This ratio inversion was associated with increased regulatory T-cells (Treg, FOXP3+) and decreased interleukin (IL)-17 producing cells (Fig. 1a), an immunological profile strikingly similar to that described for HIV-1 infected individuals.

These gut disturbances were accompanied by an increase in serum markers usually linked with high levels of microbial translocation, assessed as previously described [5]: plasma lipopolysaccharide (136 pg/ml), serum lipopolysaccharide binding protein (1.2 μg/ml) and soluble-CD14 (3.2 μg/ml), as well as systemic immune activation (Fig. 1c). Thus, our findings support a contribution of microbial translocation to HIV-2 pathogenesis, adding to this ongoing debate [5,6].

Local viral replication is considered a main determinant of HIV-1 associated mucosal disturbances. We found expression of HIV-2 Gag protein in different colon segments (Fig. 1d), which supports a significant degree of mucosal HIV-2 replication, despite the low viremia. This is in line with our previous findings of ongoing viral replication in HIV-2 patients with undetectable circulating virus, and similar levels of cell-associated viral burden in the two infections [3].

ART (emtricitabine/tenofovir/saquinavir/ritonavir) was initiated with clear virological (undetectable viremia) and immunological responses (845 $CD4^+$ T-cells/μl, 25.5%;

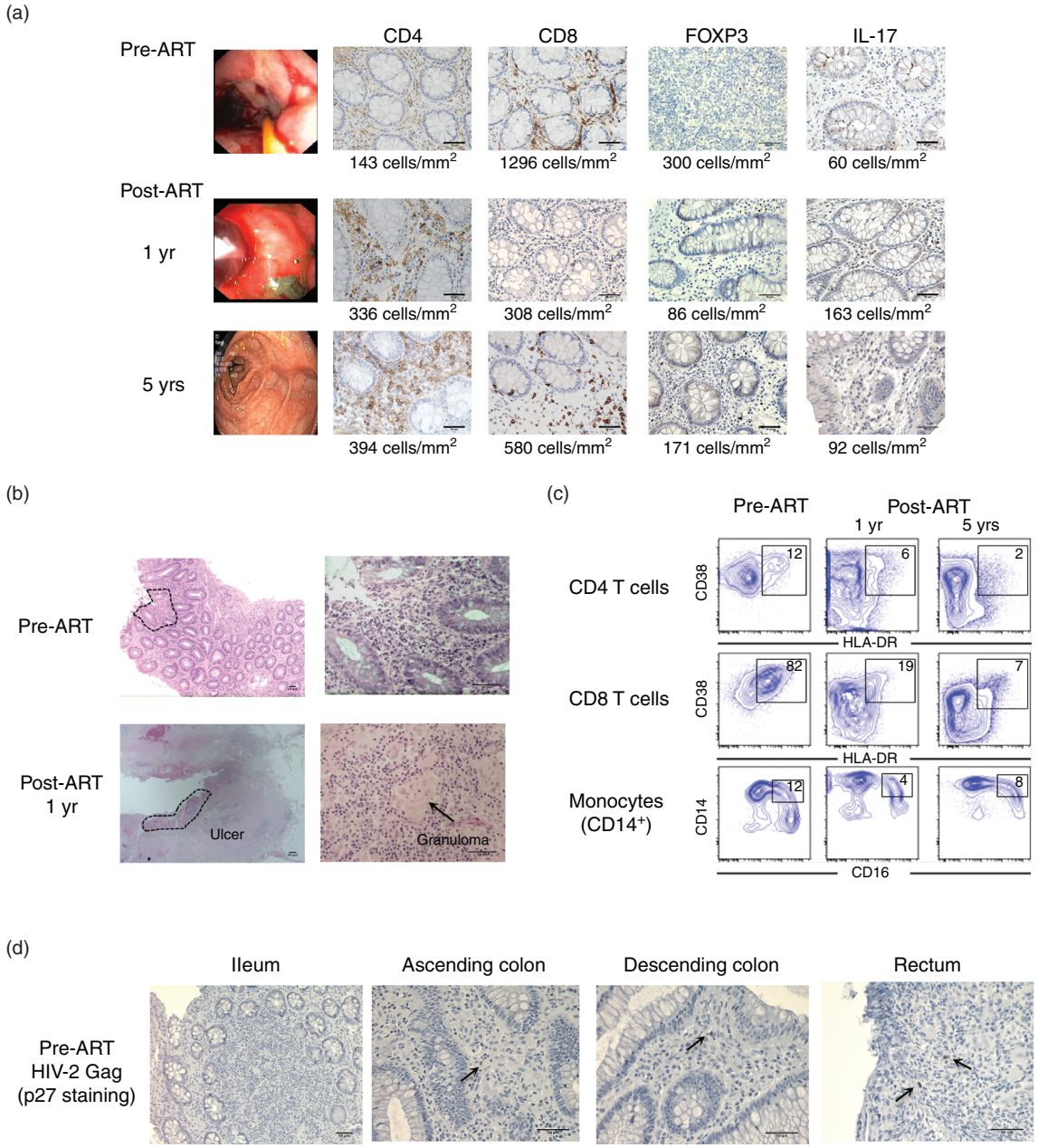

Fig. 1. Gut disturbances in an HIV-2 infected patient, and their recovery upon antiretroviral therapy. (a) Longitudinal evaluation of sigmoid mucosa: colonoscopy images and lamina propria lymphocyte populations assessed by immunohistochemistry (reference healthy controls' counts/mm^2: CD4$^+$: 501 ± 56, n = 19; CD8$^+$: 202 ± 36, n = 19; FOXP3+: 69 ± 19, n = 17; IL-17+: 123 ± 26, n = 15). (b) Haematoxylin-eosin staining of colon biopsies collected pre-antiretroviral treatment (ART) and of surgical piece (1 year post-ART). Dashed lines highlight inflammatory infiltrates and ulcerated regions. (c) Immune activation markers assessed by flow cytometry, numbers inside gates indicate frequency (reference healthy controls' frequencies, n = 16: 1.2 ± 0.1 and 4.4 ± 1.4 of HLA-DR+CD38+ within CD4$^+$ and CD8 T-cells, respectively; 4.6 ± 0.7 of CD16+CD14 bright cells within monocytes). (d) Levels of viral replication in the gut pre-ART assessed by immunohistochemistry against HIV-2 Gag (anti-SIV p27 that cross-reacts with HIV-2 p26, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from Dr P. Szawlowski). Arrows indicate positive cells. Scale bars = 50 μm.

1-year post-ART), accompanied by a progressive decline in systemic activation markers (Fig. 1c). Notably, our patient presented a marked delay of puberty (bone age of 12.5 years), and, similar to HIV-1 infected children, an interstitial lymphoid pneumonia that fully resolved with therapy.

Conversely, diarrhoea persisted with exacerbation of inflammatory histologic findings (transmural chronic inflammatory infiltrate with lymphoid aggregates, sparse noncaseating granulomas and mucosal architectural distortion), mimicking Crohn's disease, leading to the development of rectal stenosis that required endoscopic

dilatations and segmental resection (Fig. 1a,b). In spite of these disturbances, there was a recovery of lamina propria lymphocyte imbalances (Fig. 1a), as well as lack of evidence of HIV-2 replication (Gag expression, data not shown). It is plausible that this paradoxical evolution represents a form of late immunological reconstitution disease because, at that time, there was already a recovery of mucosal $CD4^+$ T-cells and IL-17 production (Fig. 1a). Gut manifestations disappeared after prolonged ART, with sustained immunological response, both in gut mucosa (Fig. 1a) and peripheral blood (716 $CD4^+$ T-cells/μl, 48.1%; 5 years post-ART).

Thus, our results represent a rational basis for starting ART in HIV-2 infection irrespectively of viremia. There are currently no randomized trials addressing ART in HIV-2 infected individuals [1]. Longitudinal studies suggest that HIV-2 infected patients have a limited response to ART, specifically a reduced $CD4^+$ T-cell recovery as compared with HIV-1 [2,3]. This might be related to virologic inefficacy, as antiretroviral drugs have not been specifically designed for HIV-2, and genotypic and phenotypic resistance studies are limited precluding an adequate choice of the best regimens [1]. Alternatively, the prolonged course of HIV-2 disease might lead to irreversible damage of secondary lymphoid organs, thereby preventing complete immunological recovery.

In conclusion, this is the first study of mucosal $CD4^+$ T-cell depletion with loss of IL-17 producing cells in HIV-2 infection, which was shown to recover upon suppression of ongoing low-level viral replication with ART. Our results represent an argument in favour of treating HIV infection in the context of reduced viremia.

Acknowledgements

S.M.F., A.R.P., C.F. and RT performed research; S.M.F., L.C. and S.E.P. did the collection of clinical data; S.M.F., R.M.M.V. and A.E.S designed the study, analysed data and wrote the article.

This work was supported by the Fundação para a Ciência e Tecnologia (FCT), and the Programa Operacional Ciência e Inovação 2010 (PIC/IC/82712/2007 to A.E.S.). S.M.F., A.R.P. and R.T. received FCT scholarships.

Conflicts of interest

There are no conflicts of interest.

Susana M. Fernandes[a,b], Ana R. Pires[a], Cristina Ferreira[c], Rita Tendeiro[a], Luís Correia[d], Sérgio E. Paulo[e], Rui M.M. Victorino[a,b] and Ana E. Sousa[a], *[a]Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, [b]Clínica Universitária de Medicina II, [c]Serviço de Anatomia Patológica, [d]Serviço de Gastroenterologia, and [e]Serviço de Doenças Infecciosas, Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Lisboa, Portugal.*

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Received: 10 October 2013; revised: 15 October 2013; accepted: 15 October 2013.

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DOI:10.1097/QAD.0000000000000114

Annex 2 – Published articles related to the Thesis work

infecção & sepsis

Contributo do estudo de infecção pelo HIV-2 para a compreensão da imunodeficiência HIV/SIDA

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Resumo

A infecção pelo HIV-2, é um modelo de doença atenuada, caracterizada pela perda lenta de linfócitos T CD4 e progressão muito lenta para Síndrome de Imunodeficiência Humana (SIDA). É nosso objectivo rever os estudos comparativos entre doentes infectados pelo HIV-1 e HIV-2 que permitiram demonstrar que: a) a activação persistente do sistema imunitário é o principal factor determinante da depleção dos CD4 quer na infecção pelo HIV-1 quer pelo HIV-2; b) há replicação viral mantida em indivíduos HIV-2+ com virémia indetectável, sugerindo que baixos níveis de replicação viral possam contribuir para uma inflamação crónica progressiva; c) a capacidade de renovação do sistema imunitário é um factor determinante da progressão da doença, conforme ilustrado pela melhor preservação de respostas a citocinas homeostáticas e da actividade tímica em doentes HIV-2+; d) um equilíbrio mais favorável com o hospedeiro depende de respostas da imunidade inata, de acordo com a reduzida produção de interferão-α in-vivo observada em doentes HIV-2+; e) mecanismos supressores, como as vias de sinalização inibitórias (PD-1/PD-L1), contribuem para limitar a activação crónica associada ao HIV. Estes estudos além de contribuirem para a compreensão da patogénese da infecção HIV/SIDA, são importantes para uma melhor definição das directivas do tratamento antirretroviral dos doentes infectados pelo HIV-2.

Palavras Chave:
HIV-2; HIV/SIDA; Activação imunológica crónica; reservatórios virais.

Introdução

A história natural da infecção pelo HIV foi alterada significativamente pela introdução de terapêutica antirretroviral (TAR), com extraordinário impacto na morbilidade e mortalidade. Um estudo recente estima que a sobrevida dos indivíduos infectados pelo HIV-1 é apenas cerca de 8 anos inferior aos seronegativos[1]. No entanto, continua a ser impossível interromper a terapêutica, uma vez que esta se associa a uma subida da viremia para níveis pré-terapêutica[2]. São também ainda controversas as indicações para o início de TAR. Para a resolução destes problemas é fundamental um melhor conhecimento da patogénese da infecção HIV. Da mesma forma, é consensual que o desenvolvimento de vacinas quer profiláticas, quer terapêuticas depende de uma melhor compreensão dos mecanismos envolvidos na resposta ao HIV.

Desde o inicio de epidemia que se reconhecem subgrupos de doentes infectados pelo HIV-1 com diferentes ritmos de progressão para SIDA, desde não progressores (mais de 10 anos de infecção mantendo CD4 superiores a 600 células/uL) a progressores rápidos (critérios de SIDA em menos de 2 anos), bem como raros indivíduos capazes de controlar a viremia na ausência de TAR. O estudo das respostas imunológicas nestes vários grupos de doentes tem sido importante para a compreensão da imunopatogenese associada ao HIV.

A infecção pelo HIV-2 é um modelo natural de doença atenuada, caracterizado por uma perda lenta de linfóctios T CD4 e ausência de virémia significativa, mesmo em fases avançadas de doença[3]. Provavelmente, devido à reduzida virémia a taxa de transmissão é muito baixa, pelo que esta infecção se manteve geograficamente confinada a uma região da África Ocidental, com epicentro na Guiné-Bissau. Portugal, devido ao seu relacionamento histórico com Guiné-Bissau e Cabo Verde, é o único país não africano com uma prevalência significativa de infecção pelo HIV-2 (4% dos casos reportados)[4].

O HIV-2 não teve impacto significativo na mortalidade mesmo em países da África Ocidental onde o HIV-2 é endémico, apesar dos doentes apresentarem uma perda lenta progressiva dos linfócitos T CD4, e um espectro de manifestações clínicas semelhante à infecção pelo HIV-1[3,5]. A carga viral plasmática é reduzida ou indetectável ao longo de todo o curso da doença pelo HIV-2, apesar do número de células infectadas, estimado pelos níveis de DNA proviral, ser semelhante à infecção pelo HIV-1. A identificação dos factores que determinam os níveis reduzidos de replicação viral em doentes HIV-2+ será importante para identificar novas estratégias de controlo dos reservatórios virais.

Assim, a comparação de indivíduos infectados pelo HIV-2 e pelo HIV-1 tem gerado contributos importantes para a compreensão da patogénese da infecção HIV/SIDA. É objectivo deste texto discutir alguns destes contributos, com particular atenção a potenciais factores determinantes da depleção mais lenta de linfócitos T CD4 na infecção pelo HIV-2, e desta forma ilustrar a enorme mais valia do estudo destes doentes. Este modelo natural de doença tem sido relativamente pouco explorado. Portugal, dada a prevalência elevada de infecção pelo HIV-2, tem condições únicas para o desenvolvimento de investigação nesta área.

Infecção pelo HIV-2 e hiper-activação imunológica

A comparação de grupos de doentes infectados pelo HIV-1 e pelo HIV-2, com o mesmo nível de perda de CD4 revelou níveis semelhantes de activação de linfó-

citos T CD4 e CD8, medida pela percentagem de células que expressam marcadores de activação celular (HLA-DR, CD38, FAS e CD69), assim como números semelhantes de células em ciclo celular (Ki67+)[6]. Uma vez que a virémia era indetectável na maioria dos doentes HIV-2 estudados, estes dados sugerem que a perda de linfócitos T CD4 está directamente relacionada com o estado de híper-activação do sistema imunitário e só indirectamente com a virémia[7].

O reconhecimento da hiperactivação crónica, subjacente à infecção pelo HIV, como causa directa de imunodepressão representou uma mudança de paradigma na compreensão da patogénese desta infecção. Este estado inflamatório facilita a replicação viral, induz modificações celulares que alteram a sua função, altera o tráfico das células entre o sangue e os tecidos, danifica a arquitectura dos órgãos linfóides secundários e deste modo promove a perda progressiva de linfócitos T "naive" quer CD4 quer CD8[8].

Por outro lado, o nosso grupo demonstrou que apesar dos níveis de híper-activação imunitária serem semelhantes para o mesmo grau de deplecção de linfócitos T CD4, os mecanismos potencialmente envolvidos no controlo desta activação parecem estar melhor preservados na infecção pelo HIV-2[9]. A este respeito, identificou-se um aumento da expressão de moléculas inibitórias (PD-1 e PDL-1) nas células T dos indivíduos infectados pelo HIV-2, o que pode contribuir para limitar os efeitos patogénicos associados ao estado de activação imunitária[10].

Infecção pelo HIV-2 e Mecanismos de Homeostasia dos Linfócitos T

As células T têm origem no timo. Embora durante a puberdade haja uma clara involução do timo, é hoje reconhecido que há actividade tímica ao longo de toda a vida. Esta actividade tímica residual é particularmente crítica em situações de linfopénia, representando um potencial alvo terapêutico para reconstituição imunológica[11].

A perda de produção tímica traduz-se numa diminuição progressiva do número de linfócitos T "naive", e pode ser estimada pela medição de produtos que resultam do rearranjo do receptor de células T no timo (TREC)[12]. Nos indivíduos infectados pelo HIV-1 este processo de envelhecimento é ainda mais pronunciado, devido ao atingimento directo do timo pelo vírus[12,13]. Em contraste, durante a infecção pelo HIV-2 parece haver uma resposta compensatória do timo, que se traduz numa melhor função tímica em indivíduos HIV-2 positivos não só, em comparação com doentes infectados pelo HIV-1, mas também com indivíduos saudáveis da mesma idade[14].

Para além da produção *de novo* de células T no timo, o número de linfóctios T depende também de citocinas que induzem sinais de sobrevivência nestas células, particularmente nos gânglios linfáticos. Uma das citocinas homeostáticas mais importante é a Interleucina (IL)-7[15], cujos níveis séricos aumentam em situações de linfopenia, traduzindo uma resposta homeostática. A resposta a esta citocina é em parte regulada pela expressão do seu receptor (IL-7Rα) à superfície das células.

Em indivíduos infectados pelo HIV-2, o nível sérico de IL-7 correlaciona-se fortemente com a diminuição de linfócitos T CD4. Por outro lado, este aumento de IL-7 associa-se a um aumento da expressão do IL-7Rα nos linfócitos T CD4 "naive" dos doentes infectados pelo HIV-2, o que não ocorre nos doentes infectados pelo HIV-1, sugerindo uma melhor preservação das respostas homeostáticas à linfopenia[16].

Outra das citocinas fundamental para a expansão e sobrevida dos linfócitos T é a IL-2, cuja a resposta de activação celular é dependente da expressão, da cadeia α do seu receptor (CD25).

Durante a infecção pelo HIV está descrito uma desregulação das respostas à IL-2. Ao contrário do que ocorre na infecção pelo HIV-1, na infecção pelo HIV-2 a capacidade para produzir esta citoquina está relativamente preservada[17]. Por outro lado, demonstrou-se a expansão de linfócitos T CD4 exprimindo CD25 e produzindo IL-2 em doentes infectados pelo HIV-2, o que poderá contribuir para uma melhor preservação dos linfocitos T CD4, e desta forma para respostas antivíricas mais eficazes[18]. No que respeita, às células T CD4 especificas contra o HIV, trabalhos do nosso grupo documentaram uma melhor preservação da qualidade da resposta especifica anti-Gag dos linfocitos CD4 em doentes infectados pelo HIV-2, avaliada pela capacidade destas células produzirem IL-2[19].

Tendo em conta a importância destas citocinas na homeostasia dos linfócitos T CD4, tem sido estudado o impacto do seu uso terapêutico como complemento à TAR na recuperação imunológica[20]. Embora os resultados dos ensaios com a IL-2 sejam controversos[21], os estudos de fase II com IL-7 são promissores, particularmente no que respeita à reconstituição das células T CD4 "naive"[22,23].

HIV-2 e controlo dos reservatórios virais

Tal como na infecção pelo HIV-1, o principal alvo de infecção pelo HIV-2 são células T CD4 de memória, CCR5 positivas[24]. No entanto, apesar da ausência de viremia, o número de células infectadas estimado pela quantificação de DNA proviral é semelhante em ambas as infecções. Além disso, a documentação de transcritos de gag mRNA em células do sangue periférico de doentes infectados pelo HIV-2 sugere a existência de replicação viral persistente nestes doentes[25], o que poderá contribuir para a activação imunitária. Como discutido adiante, será importante estudar os tecidos linfóides de doentes infectados pelo HIV-2 para identificar os mecanismos que determinam a ausência de carga viral plasmática.

Estes dados são ainda particularmente relevantes para a discussão de directivas clínicas de TAR para o HIV-2, dada a escassa informação que os clínicos têm disponível para basear as suas decisões terapêuticas.

O duplo papel do IFN-α na patogénese da infecção HIV/SIDA

O IFN-α é uma das principais citocinas com actividade antivírica, tendo também a capacidade de activar múltiplas componentes do sistema imunitário, nomeadamente células NK, células T e células dendríticas[26]. Assim apesar de fundamental nas respostas antivirais, quando esta citocina é produzida cronicamente em grandes quantidades tem um papel deletério, contribuindo para o estado de híper-activação imunitária subjacente à progressão da imunodeficiência.

Na infecção crónica pelo HIV-2 há ausência de aumento de produção *in vivo* de IFNα avaliada pela expressão de genes induzidos por esta citocina, ao contrário do que se observa na infecção pelo HIV-1,

o que é favor de um papel deletério de níveis elevados de IFNα [27,28].

HIV-2 e erosão do sistema imunitário

O tempo de infecção nos doentes infectados pelo HIV-2 é frequentemente superior a 20 anos na ausência de manifestações clínicas, associando-se no entanto a uma perda lenta progressiva de linfócitos T CD4. Embora não existam ensaios clínicos controlados de TAR na infecção pelo HIV-2, os vários estudos de coorte existentes indicam que a recuperação imunológica após inicio de TAR nestes doentes é geralmente reduzida, sugerindo a possibilidade de um deficit irreversível do sistema imunitário[29].

Neste sentido, o nosso grupo demonstrou uma depleção marcada de células B de memória mais acentuada em doentes HIV-2 do que HIV-1, apesar de ausência de virémia e do mesmo estádio de depleção de células T CD4 [30]. Estes resultados apontam para uma possível erosão dos gânglios linfáticos, fundamentais para a homeostasia das células B de memória.

Conclusões e Perspectivas futuras

Em resumo, a comparação entre doentes infectados pelo HIV-1 e HIV-2 permitiu revelar que: a) a activação persistente do sistema imunitário é o principal factor determinante da depleção dos CD4 quer na infecção pelo HIV-1 quer pelo HIV-2; b) há replicação viral mantida em indivíduos HIV-2+ com virémia indetectável, sugerindo que baixos níveis de replicação viral possam contribuir para uma inflamação crónica progressiva; c) a capacidade de renovação do sistema imunitário é um factor determinante da progressão da doença, conforme ilustrado pela melhor preservação de respostas a citocinas homeostáticas e da actividade tímica em doentes HIV-2+; d) um equilíbrio mais favorável com o hospedeiro depende de respostas da imunidade inata, de acordo com a reduzida produção de interferão-α in-vivo observada em doentes HIV-2+; e) mecanismos supressores, como as vias de sinalização inibitórias (PD-1/PD-L1), contribuem para limitar a activação crónica associada ao HIV.

Estes resultados ilustram o potencial deste modelo natural de doença HIV atenuada para a compreensão da patogénese da SIDA.

É importante realçar que não existem dados sobre os tecidos linfóides na infecção crónica pelo HIV-2, estudo que será fundamental para a compreensão de múltiplos aspectos relacionados com a história natural desta infecção, tal como referido anteriormente.

A identificação de estratégias que limitem a imunopatologia associada ao HIV e promovam a reparação dos tecidos constitui uma prioridade na investigação em HIV/SIDA. Adicionalmente, estes estudos poderão identificar novas estratégias para o controlo dos reservatórios virais na infecção HIV e para preservar/reconstituir imunocompetência. Finalmente, dada a importância da epidemia pelo HIV-2 em Portugal, a criação de uma rede que permita o estudo integrado dos dados clínicos, imunológicos e virológicos dos doentes infectados pelo HIV-2 trará importantes dividendos para o seguimento clínico destes doentes.

Agradecimentos: SMF recebe uma bolsa da Fundação para a Ciência e Tecnologia SFRH/SINTD/60108/2009.

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